Rijksuniversiteit Groningen

EFFECTS OF OVERLAP OF HYDRATION SHELLS ON NONCOVALENT INTERACTIONS IN AQUEOUS SOLUTION

A KINETIC STUDY

Proefschrift

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te Amersfoort

Promotor: Prof. Dr. J.B.F.N. Engberts

voor mama en papa

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Lisette.

Contents

CHAPTER 1

An Introduction into the Solution Properties of Biological Molecules in Water

1.1 Water; vitally important for biological processes

Many people consider liquid water as a normal liquid, presumably because of its abundance and omnipresence in everyday life. However, to many scientists, water is an unusual liquid with very characteristic properties, such as a very high cohesive energy density due to its 3-dimensional hydrogen bond network (reflected by its relatively high boiling point), a relatively low density, a high relative permittivity and a low polarisability compared to other organic solvents. The properties of water and aqueous solutions have been extensively studied over the past century and comprehensive reviews have been written[1,2].

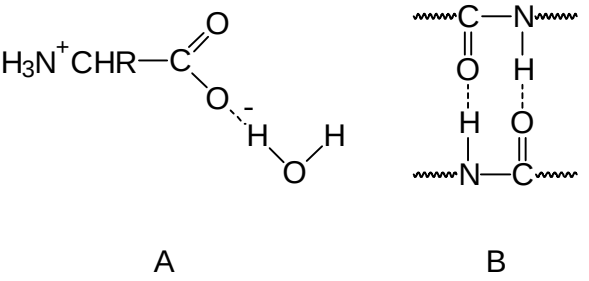
Water is an indispensable substance for living systems. Biological processes and the formation of biological structures, such as the folding of proteins and the formation of lipid cell membranes, are uniquely mediated by the solvent water. These processes are brought about by noncovalent interactions. Particularly, hydrophobic interactions between apolar groups play an important role in the molecular recognition processes of biological molecules in water.

In this introduction, the noncovalent interactions which play a role in aqueous solutions containing (biological) molecules will be discussed briefly, with the emphasis on hydrophobic interactions. These form the underlying basis for the discussion of the results described in this thesis, which deals with intermolecular noncovalent interactions. Since the focus of this thesis is on aqueous solutions of α-amino acids, solute-solvent (solute hydration) and solute-solute interactions involving these solutes will be described in more detail. In the final section, the aim of the study is outlined and the contents are previewed.

1.2 Noncovalent interactions of importance in biochemistry

Noncovalent forces, unlike covalent forces, do not involve electron pairing effects, but are brought about by changes in electron density distribution. The strength of noncovalent interactions depends on the solvent, the molecular structure of the solute and the intermolecular distance between the interacting species.

Notwithstanding the fact that noncovalent forces are much weaker than covalent bonds (*e.g*. compare the formation of a hydrogen molecule from the reaction of two hydrogen atoms (-400 kJ/mol) with the formation of the noncovalent H-bond in water (-40 to -10 kJ/mol)), many molecular recognition processes are governed by noncovalent interactions. The base-stacking and hydrogen bonding in the DNA double helix and the H-bonding in the peptide α-helix are examples where noncovalent forces determine shape. Noncovalent interactions also constitute the basis for supramolecular host-guest complexes[3]. In aqueous solution, noncovalent interactions are responsible for the stabilisation of proteins[4], the assembly of lipids in biomembranes[5] and surfactant aggregation[6]. The most important forces which determine the strength and specificity of noncovalent (bio)molecular associations in aqueous solutions are electrostatic, London dispersion and hydrophobic forces. Electrostatic interactions are strong, long-range and directional and comprise ion-ion, ion-dipole and dipole-dipole interactions, such as H-bonding interactions (Scheme 1.1). The importance of H-bonding in biological structures has been extensively described[7].



Scheme 1.1 *H-bonding in solute-solvent (A), and in solute-solute (B) interactions.*

Dispersion forces are short-range and can occur between molecules without permanent dipoles. They can become quite substantial when the polarisability (number of polarisable electrons) of the molecules increase. They play a role in, among others, the stabilisation of biomembranes and protein-ligand binding.

Hydrophobic interactions are solvent-induced interactions between apolar moieties and are an outstanding feature of aqueous solutions. They are driven by the urge of water in overlapping hydrophobic hydration shells to regain the properties of water in the bulk. The interpretation of the thermodynamic observations of hydrophobic hydration and hydrophobic interactions has been reconsidered in the last decade. The negative entropy and enthalpy change, and the positive change in heat capacity upon dissolution of apolar solutes in water led to the introduction of the concept of the 'iceberg' model in 1957[8]. This model explained the large entropy loss upon dissolution of apolar gases in terms of structuring of water molecules in the near vicinity of the solute. The enthalpy gain was explained by increased water-water hydrogen bonding interactions. A few years later, the concept of hydrophobic interactions was introduced[9]. The entropy-driven interaction between apolar moieties was explained by a release of structured water

molecules as a result of destructive overlap of hydrophobic hydration spheres. For several decades, researchers have been inspired by these interpretations. However, in the last ten years, serious doubts about these interpretations have developed, due to the use of more advanced experimental and computational techniques. Recent opinions and facts about these new developments have been reviewed[10]. Computational techniques have shown that water does not undergo a substantial structural enhancement (*i.e*. increased H-bonding) in the hydration shell of apolar solutes[11], but largely maintains its original structure by accommodating the apolar solutes in its 3-D H-bond network, without sacrificing a significant number of hydrogen bonds. A parallel orientation of O-H bonds of the water molecules to the apolar solute surface was suggested to be favourable and was experimentally proven by neutron scattering studies[12]. To date, the thermodynamic observations accompanying the dissolution of an apolar solute in water are explained in terms of London dispersion interactions between water and the apolar solute, which can be quite substantial[13], leading to the favourable enthalpy of dissolution, and to a reduction in the configurational degrees of freedom of water[14,15,16], causing the unfavourable entropy of solvation.

In view of the opinions postulated above, it must be stressed that hydrophobic interactions are not so much driven by favourable interactions between the apolar solutes, but that they are rather the consequence of water favouring interactions with itself.

The distinction between bulk and pairwise hydrophobic interactions should be noted. Bulk hydrophobic interactions, leading to surfactant aggregates, such as micelles and lipid bilayers, result from the fact that insufficient water is available for the formation of independent hydration shells, so that association cannot be prevented. These aggregates form with a high cooperativity. London dispersion energy contributes significantly to their stabilisation. Pairwise hydrophobic interactions (1:1 interactions), on the other hand, do take place via destructive overlap of independent hydration spheres. London dispersion interactions play a much less important role, since for these direct interactions, dehydration of the solutes is required. Currently, it is thought a misinterpretation to represent pairwise hydrophobic interactions as a static phenomenon in aqueous solution. The apolar molecules do not ‘search’ for each other and then ‘stick’ together incessantly in aqueous solution, but rather meet in ‘hydrophobic encounters’ (the ‘hydrophobic kiss’). On average they spend more time in close contact than that they are ideally distributed in the aqueous solution.

1.3 Noncovalent interactions in protein folding and stability

Based on several experimental observations, it is generally accepted that protein folding is driven by hydrophobic interactions between apolar α-amino acid side chain residues[9,17,18,19]. Nonetheless, discussion concerning the importance of hydrophilic interactions in the folding processes is continuing[20,21]. Particularly, the interpretation of the thermodynamics of amide-amide and amide-water interactions has been a problematic issue[22].

Intramolecular noncovalent interactions are responsible for the unique three-dimensional structure into which proteins fold in aqueous solution. Noncovalent interactions that contribute to the stability of the folded protein include H-bonding interactions between peptide groups which stabilise secondary structures (α-helices and β-sheets), salt-bridges (ion-paring) between charged residues and London dispersion interactions between apolar residues. The stability of the folded structure, however, is only marginal. The Gibbs energy difference between the folded and unfolded state of a protein is typically[23] only 20-50 kJ mol^{-1}, which is, for comparison, equivalent to only a few hydrogen bonds. Since there are many intramolecular hydrogen bonds in the folded state there must be noncovalent interactions destabilising the native state. The main destabilising factor is the greater conformational entropy of the unfolded state, though its value is unknown. Amide-water hydrogen bonding interactions also destabilise the native state, but as was mentioned above, there exists uncertainty about the relative contributions of amide-water and amide-amide interactions towards the stability of the protein. Overall, the small net stability of a protein can be ascribed to large compensating entropic and enthalpic contributions.

The thermodynamic behaviour of proteins has been extensively discussed[17,24]. However, a lack of experimental information of the magnitude of the contributions of various noncovalent interactions to protein stability remains.

1.4 Hydration of polyfunctional molecules

A molecule immersed in water gives rise to the formation of a so-called hydration sphere[25,26], in which water (hydration water) has different structural properties from those of bulk water. In the previous section we discussed the hydration water of apolar groups in terms of hydrophobic hydration. Biological molecules are polyfunctional molecules which contain, apart from apolar groups, also hydrophilic

moieties, and therefore give rise to the formation of hydrophobic *and* hydrophilic hydration shells in aqueous solution. The interactions of polar groups with water (hydrophilic hydration) are strong and long-range and take place predominantly via electrostatic (hydrogen bonding) interactions[7]. The presence of both hydrophilic and hydrophobic hydration regions around a polyfunctional molecule makes the study of the hydration of polyfunctional molecules a complex matter. The intramolecular hydration shells can interact and in a destructive way when the water structure in the hydration layers is different and therefore incompatible. This interaction will have important consequences for solute-solute interactions. In order to obtain information about the Gibbs energetics of the noncovalent interactions involving biological molecules, the hydration properties of these solutes need to be investigated. Below, it is shortly outlined how solute hydration can be studied and subsequently the hydration properties of the polyfunctional α-amino acids are discussed.

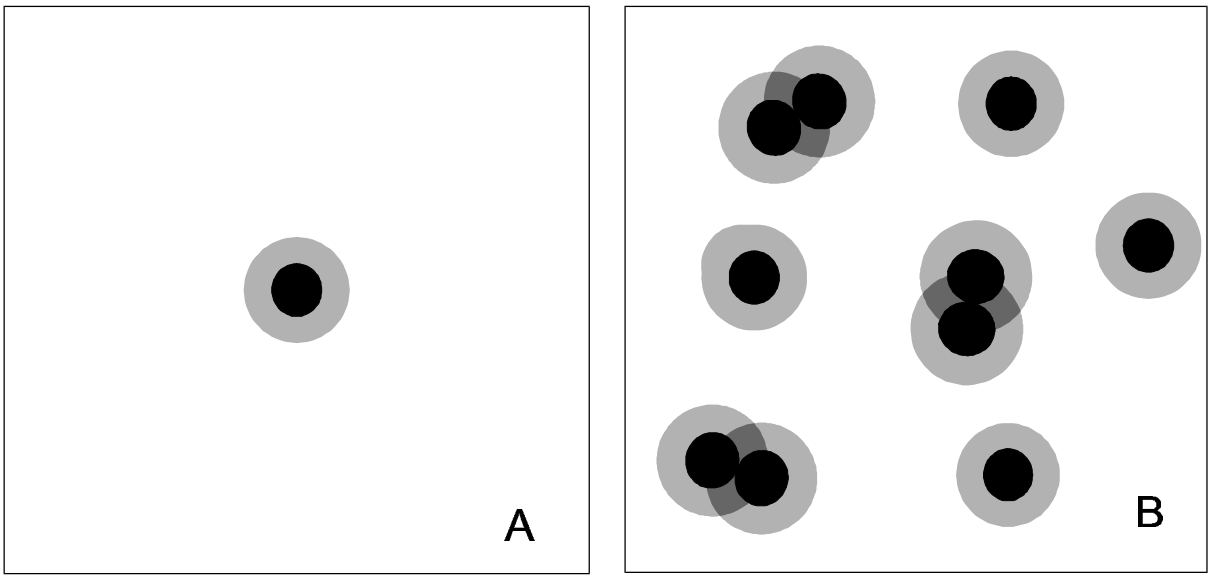
1.4.1 Studying solute hydration

Principally, there are two ways in which information can be obtained about the hydration characteristics of a solute:

1) By studying *ideal* solutions (Scheme 1.2A), *i.e*. where the solute is present at infinite dilution and apart from solvent-solvent interactions, information about *solute-solvent* interactions is obtained.
2) By studying *nonideal* solutions (Scheme 1.2B), *i.e*. where, in addition to the interactions mentioned under 1, also *solute-solute* interactions play a role. Solute-solute interactions are presumed to take place via overlap of the individual hydration spheres.

Solute-solvent interactions can be studied directly by measuring Gibbs energies of transfer of the solute from the gas phase to water. By extrapolation to zero concentration Gibbs energies of hydration are obtained. The solvation Gibbs energies of biological molecules are increasingly studied by theoretical (computational) methods. The goals of these studies are either to produce values that are in agreement with experiment or to be predictive. The power of solvation Gibbs energy calculations employing molecular dynamics and Monte Carlo methods in biochemical phenomena has been demonstrated[27]. Recently, neutron scattering techniques have shown to be a reliable method for the study of solute hydration[12]. Other methods which are employed to obtain information about the solute hydration where solute-solute interactions do not interfere, are the measurements of the partial molar solute properties at infinite dilution, such as the partial molar volume,

heat capacity and compressibility. These properties are different for a pure solute



Scheme 1.2 *Pictorial representations of (A) an ideal aqueous solution and (B) a nonideal aqueous solution. (Solutes: black, hydration shells: grey.)*

and a solute in aqueous solution. This difference is a combination of an intrinsic and, of more interest, a solvation contribution.

The second approach provides a more indirect way of investigating the characteristics of the solute hydration, but the advantages are that numerous techniques can be employed and that the results are relevant for (bio)molecular association and recognition processes in water. Solute-solute interactions in water take place via overlap of the individual hydration spheres. All deviations from thermodynamic ideality of the solution can be ascribed to solute-solute interactions and can therefore be quantified by measuring the excess thermodynamic properties of the solution, *i.e.* the $X_{excess}=X_{solution}-X_{ideal}$ (where X is the measured thermodynamic property). As will be seen shortly, this method is employed in the studies described in this thesis. The thermodynamic quantity is the Gibbs energy (X=G).

1.4.2 Additivity principle of solute-solute interactions

In a thermodynamic approach first formulated by Savage and Wood[28], it was suggested that the thermodynamic parameters of interaction between two solutes in aqueous solution are a sum of the interactions between the functional groups

making up the solute molecules. Their approach (Savage and Wood Additivity of Groups, or shortly SWAG) is based on the following assumptions:

1) Each functional group in solute A interacts with each functional group in solute B.
2) Each group-group interaction makes a characteristic contribution to the solute A-solute B pairwise interaction parameter.
3) Each group interaction parameter is independent of all other functional groups and their relative stereochemistry in solute A and solute B.

The approach has been used to analyse thermodynamic data for many solutes. Additivity in Gibbs energies and enthalpies for noncovalent interactions between non-electrolytes in aqueous solution[29] has been often observed. It has also been found applicable for the analysis of interactions between electrolytes and non-electrolytes[30].

Although additivity schemes have been criticised[31], and rightly so, they are still applied in the analysis of thermodynamic solute-solute parameters.

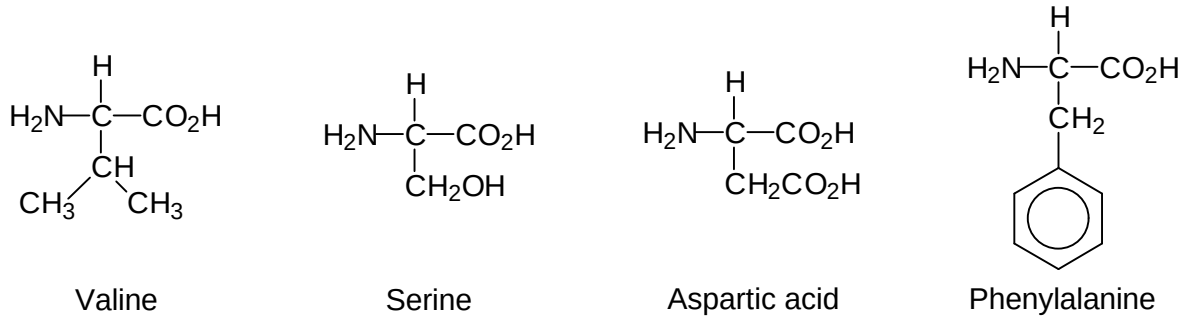
1.4.3 Aspects of α-amino acid hydration

In order to obtain more insight into the hydration of proteins and the noncovalent forces stabilising their native structure, it is necessary to study the hydration of their primary building blocks; the α-amino acids. Particularly the hydrophobic hydration of the α-amino side chains is interesting in view of the driving forces behind protein folding. Apart from this, the study of free α-amino acids is also interesting with respect to their occurrence in living organisms in the free form. The majority of these α-amino acids is present in the cytoplasm of cells, and is used for the biosynthesis of proteins. α-Amino acids can be transported over the cell membrane. In addition, they have distinct functions, for example as neurotransmitters[32].

The hydration properties of α-amino acids and α-amino acid derivatives have been studied following the two approaches described (1.4.2) and many of these studies have been reviewed[33,34]. A number of studies, which bear important conclusions with respect to the heterogeneous hydration of α-amino acids, will be discussed. Where the measured hydration properties of α-amino acids have been analysed with the group additivity approach, mention will be made. A short introduction into α-amino acid chemistry is helpful for understanding their hydration properties.

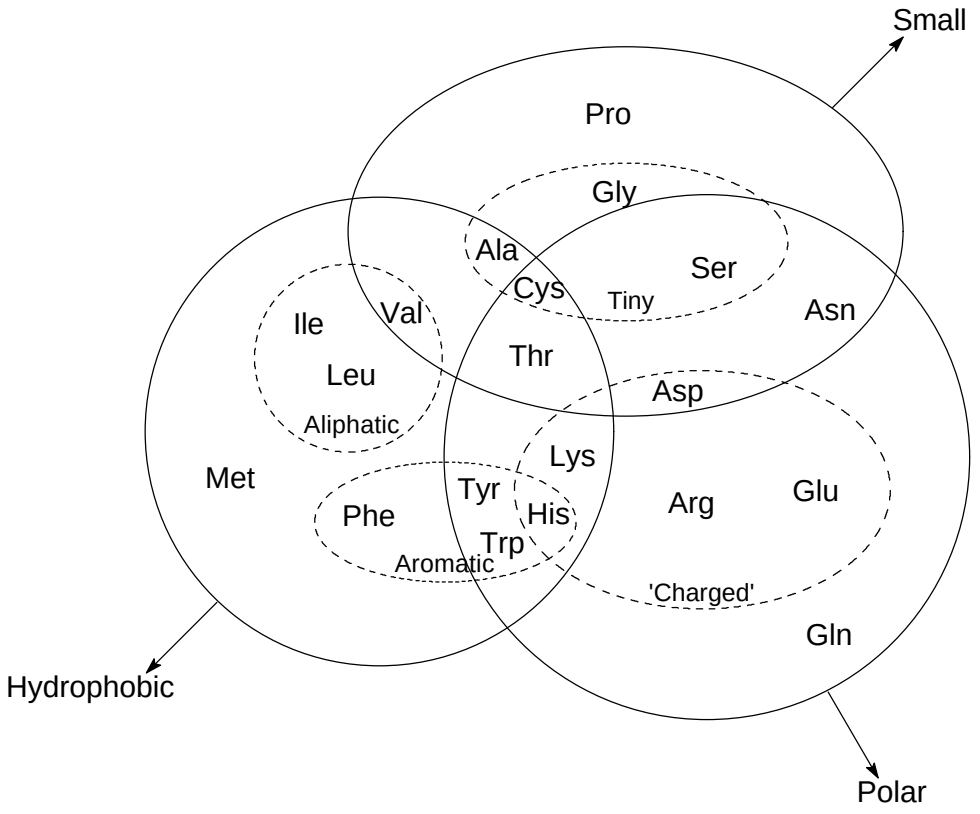
1.4.3.1 Classification and zwitterionic behaviour of α-amino acids

The 20 naturally occurring α-amino acids differ in substitution on the (chiral) α-C atom. Some examples of α-amino acids are shown in Scheme 1.3.



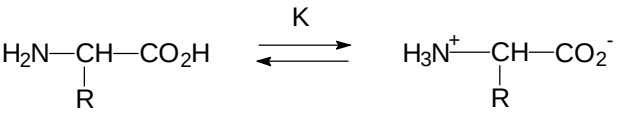
Scheme 1.3 *Examples of α-amino acids.*

According to the character of the side chain, the α-amino acids can be divided into different categories classified as aliphatic, hydrophobic, aromatic, polar or charged (see Scheme 1.4). An important feature of α-amino acids is their existence as inner salts or zwitterions in aqueous solution. How this dipolar character is affected in the



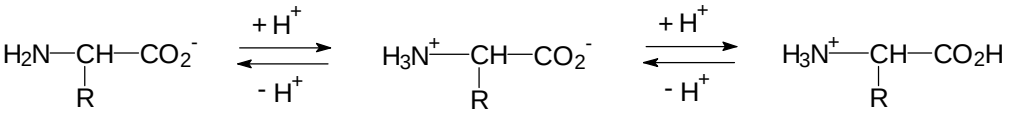
Scheme 1.4 *The 20 naturally occurring α-amino acids classified according to their side chain structure (taken from ref. 35).*

presence of a solvent is nicely illustrated by the equilibrium constants for the tautomeric equilibrium between the neutral and zwitterionic forms of glycine in the gas phase and in water resulting from intramolecular proton transfer:



In the gas phase[36], K can be estimated to be 10^{-21} whereas in water[37] the equilibrium constant is ca. 4 x 10^{6} at ambient temperature. In water, electrostatic ion-dipole interactions stabilise the zwitterions.

As a consequence of their amphoteric character, α-amino acids can either act as acids or bases:



1.4.3.2 Solute-solvent interactions involving α-amino acids

Due to the extremely low volatility of α-amino acids, determination of transfer parameters from the gas phase to water is very difficult. Instead, model compounds have been used[38], but these lack the zwitterionic character which is so determinant for their solvation behaviour. The Gibbs energies of transfer of α-amino acids from an apolar solvent to water are probably more informative. They are relevant for protein folding studies since they represent the transfer of an α-amino acid residue from the interior of a protein to water. Tanford[39] determined the Gibbs energies of transfer from ethanol to water which led to a hydrophobicity scale for α-amino acids[40]. Every group in the amino acid seemed to make a constant contribution to the Gibbs energy of transfer. Although this study did not include all 20 α-amino acids and the order of hydrophobicity observed is specific for the apolar solvent used[41], the study is still often referred to[40]. The relative solvation Gibbs energies for α-amino acids have also been computed[42] and the results confirmed the experimental results.

Partial molar volumes[43,44,45,46,47], heat capacities[45,48] and adiabatic compressibilities[43,49,50] for α-amino acid solutions have been measured over the past decades. Volumes and heat capacities roughly increase with increasing molar mass,

but superimposed on that relationship are effects arising from specific hydration behaviour. Isentropic compressibilities of α-amino acid solutions are reduced

Table 1.1 *Partial molar isentropic compressibilities(PMC) for some α-amino acids and amides at 25°C*[a].

Solute	10^4 x PMC ($cm^3mol^{-1}bar^{-1}$)	Solute	10^4 x PMC ($cm^3mol^{-1}bar^{-1}$)	Solute	10^4 x PMC ($cm^3mol^{-1}bar^{-1}$)
Gly	-27.0	Thr	-31.2	Val	-30.6
Ser	-29.9	Aba[b]	-21.8	Leu	-31.8
α-Ala	-25.5	Aiba[c]	-23.5	Glygly	-35.5
β-Ala	-26.4	Pro	-23.3	Glygly dkp[d]	-11.1

[a]Data from ref.43 and 50 [b]α-Aminobutyric acid [c]α-Aminoisobutyric acid [d]Diketopiperazine

compared to the compressibility of pure water and there is a tendency to become more negative for more hydrophobic α-amino acids (Table 1.1). However, this property, like the volume and heat capacity, reflects specific hydration characteristics, as is indicated by a comparison of the values for isomeric solutes.

An important effect of the zwitterionic character of α-amino acids on the hydration water, which is reflected by the partial molar properties, is the contraction of water molecules around the zwitterionic groups, called electrostriction. Electrostriction of water around the charged groups was first emphasised by Zana[51], who measured the volume changes upon protonation of alkylcarboxylates and alkylamines. It appeared that electrostriction caused by the ammonium group is large but that the carboxylate group induces only little electrostriction. Chalikian *et al*.[52] investigated the partial molar volume and compressibility changes upon ionisation of amino and carboxylate groups in α-amino acids. Ionisation of the amino group leads to a considerably larger volume contraction than ionisation of the carboxylate group, thereby supporting Zana's data[51]. The combined volume change, however, does not equal the difference in volume observed for uncharged and zwitterionic α-amino acids[44a]. From this observation, the authors concluded that the ionic hydration shells interfere intramolecularly. In α-amino acids where the charged groups are attached to the same carbon atom, electrostriction is significantly less than in cases where they are further separated. This appeared from studies in which partial molar properties for aqueous solutions containing zwitterionic α,ω-amino acids[53,54,55] have been determined. It was shown that the charged termini in α,ω-amino acids are not independently hydrated, but that they interact via overlapping hydration shells up to four intervening methylene groups in the case of the volumes and expansibilities and even six in the case of the compressibilities. A similar study for oligoglycines[56] confirmed these intramolecular interactions. In diglycine the

charged termini still interact via their hydration shells (four interposing atoms) but not in triglycine, causing a breakpoint in the plots of partial molar properties versus the number of peptide bonds. Thus, the two ionic hydration shells in α-amino acids are not independently hydrated and their reduced electrostatic interactions with the solvent are due to incompatible hydration sphere overlap. The different hydration characteristics of the ammonium and carboxylate groups were also reflected by the volume changes due to the alkyl chains which were affected differently by the two ionic groups[51] and by the fact that upon ionisation compressibility increases in the hydration shell of the amino group but decreases in the hydrated water of the carboxyl group[52]. Since these interactions are long-range, effects on the hydration of the α-substituent are inevitable. Indeed, the α-amino acid apolar group contributions to the partial molar properties differs from those of other classes of organic solutes. To return to the studies of α,ω-amino acids[53-55], it is observed that for the shorter solutes, the action on water structure is purely electrostrictive, indicating a dominance of hydrophilic over hydrophobic hydration. For the longer solutes, the hydrophobic hydration of the methylene groups is more pronounced. The hydrophobic and hydrophilic hydration shells differ not only in absolute values of density and the coefficient of compressibility but also in the temperature dependence of these values. A temperature and pressure dependent study of the partial molar properties of Gly and α-Ala[57] indicated that the hydration of Gly is completely dominated by electrostatic solute-solvent interactions and that the methyl group in α-Ala is clearly not independently hydrated, *i.e*. the hydration of the methyl group is influenced by the ionic hydration shells. Mishra *et al.*[46] determined partial molar volumes of zwitterionic α-amino acids and peptides in aqueous solution and observed deviations from apolar group additivity up to a distance of four consecutive CH_2-groups remote from the hydrophilic groups, also indicating the long-range effect of zwitterionic hydration on the contributions of apolar groups to the volumes. A study by Reading *et al*.[58] showed that the volume contribution of an α-methyl group to the partial molar volume is substantially different from that of an *N*-methyl group. Deviation of apolar group additivity was also observed in the volumetric properties of aqueous solutions containing terminally substituted α-amino acids and dipeptides[59]. The results have also been explained in terms of electrostrictive effects on water. The analysis of another type of volumetric parameter of dilute aqueous α-amino acid solutions[60] showed a significant sensitivity to the change of position of an atomic group, *i.e*. differences between the hydration of isomers and, subsequently, deviations from group additivity contribution to the measured parameter are observed.

1.4.3.3 Solute-solute interactions involving α-amino acids

Usually, solute-solute interactions are studied by heats of dilution of aqueous solutions using microcalorimetry. The dependence of the excess enthalpy on the solute concentration can be described by a molality expansion yielding virial coefficients that are a measure of the interactions between pairs, triplets, etc., of solute particles[61]. This method has also been employed for aqueous α-amino acid solutions. There are few studies that report pairwise Gibbs *energy* self-interaction parameters involving α-amino acids[62]. The Gibbs energies are small, but both the enthalpies and entropies are large and opposite in sign, a feature characteristic for aqueous solutions. Gibbs energies of interaction are negative for Gly and Ala, indicating a net attraction between these acids and positive for α-amino acids with longer apolar side chains, reflecting a net repulsion. Figure 1.1 shows that there is a rather gradual change of the parameters with alkyl chain length, indicating additive contributions of the CH_2-group to the parameters. However, when isomers are included, the linear relationships disappear, and the SWAG-approach fails.

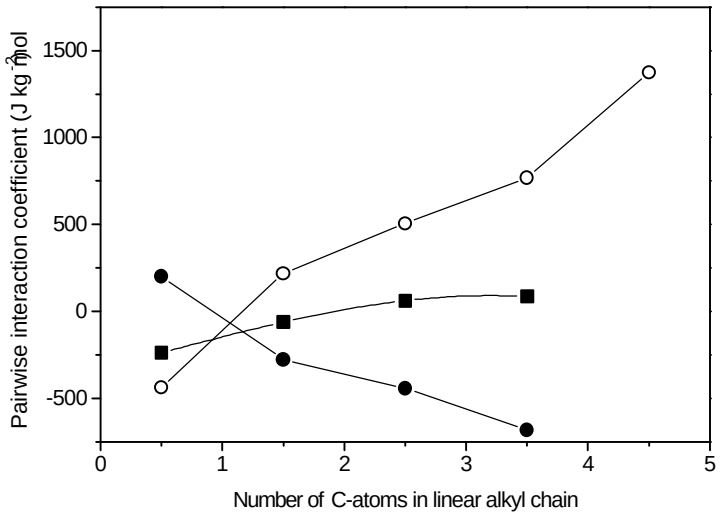


Figure 1.1 *Dependence of the g_{xx} (■), h_{xx} (O) and -Ts_{xx} (●) on the number of C-atoms in the alkyl chains of Gly, Ala, Aba, nVal and nLeu. (Data from ref.63.)*

Unfortunately, there exists some inconsistency in the data[64]. Clearly α-amino acids interact via both hydrophilic and hydrophobic interactions, suggesting a positioning to each other which allow both types of interaction to take place simultaneously. In addition to the studies of binary α-amino acid solutions several studies which deal with intermolecular interactions of α-amino acids with amides[65,66], ureas[65],salts[67,68] and polymers[69] (*i.e* ternary aqueous solutions) have been reported.

The enthalpic interaction coefficients between Gly and series of substituted amides and ureas[65,66] show the dominance of long-range electrostatic solute-solvent

interactions for Gly. Favourable enthalpic interactions with the peptide group and unfavourable interactions with the amide CH_2-groups were observed. Additivity of group interactions was observed for interactions between Gly and substituted ureas, but not for interactions with the amides. The hydrophilic hydration of Gly was also evident from interactions between poly(ethylene glycol) (PEG) and zwitterionic Gly, Ala, Val, Ile and Leu in aqueous solutions[69]. Gly (and also Ala) did not interact with the PEG methylene moieties through hydrophobic interactions, but α-amino acids with more pronounced hydrophobic moieties could. Theoretical studies also supported the hydrophilic character of Gly[70]. Care should be taken to classify Ala as hydrophilic, since its side chain affects intermolecular interactions as is shown by the clear contribution of the α-methyl group to the h_{xx}[63,71] and to the h_{xy} in the interaction with amides[66]. The question remains whether these effects should be attributed to hydrophobic interactions or whether the apolar group mediates polar group-polar group intramolecular interactions.

The enthalpic interaction coefficients between amides and ammonium methanoate where the ionic components are distinct and separate, unlike those of the amino acids, have also been determined[72]. Interestingly, the energetics of interactions with hydrophobic and peptide groups are not significantly perturbed by the proximity of the ionic groups to each other.

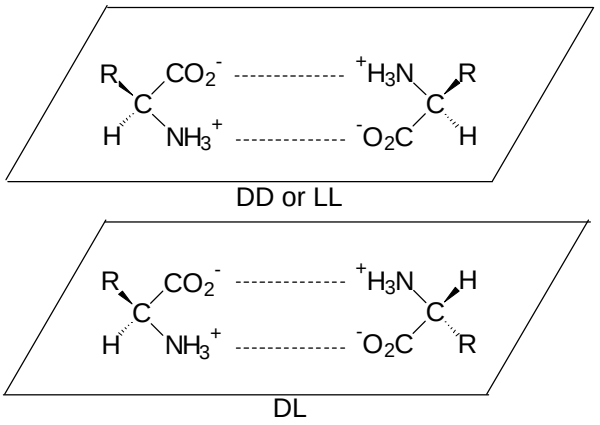
Interactions with salts[67], such as NaCl, take place primarily with the zwitterionic headgroup. The electrostrictive effect on water is thereby reduced[68]. The zwitterion-salt interaction perturbs the solvation of the α-amino acid alkyl chain up to the fourth carbon atom.

The amount of data on solute-solute interactions involving zwitterionic α-amino acids is not extensive. Most studies deal with intermolecular interactions involving aqueous solutions containing uncharged, *i.e*. protected, α-amino acids, such as *N*-acetyl amino acid amides and small peptides[73]. These are interesting with respect to biological interaction processes, particularly in view of amide-amide interactions and for peptide side chain hydration. However, for the hydration properties of *zwitterionic* α-amino acids they are less relevant.

1.4.3.4 α-Amino acid - α-amino acid interactions: chiral recognition

Although there are many examples of chiral recognition of (derivatives of) α-amino acids in a chiral apolar environment[74], chiral recognition is not expected between pairs of enantiomeric α-amino acid molecules in dilute solution in non-chiral polar solvents, because the solute-solute interactions compete with solute-solvent

interactions. This is particularly true in the case of the solvent water whose molecules interact strongly with polar groups of the solute. However, microcalorimetric evidence for chiral interaction of the D- and L-enantiomers of protected α-amino acids has been obtained[75,76,77]. The enthalpic pairwise interaction coefficients of isomers of different chirality (h_{DL}) differ clearly from the enthalpic pairwise interaction coefficients of isomers of the same chirality (h_{DD} and h_{LL}). Both polar and hydrophobic interactions contribute to these differences. Since these seem to be solvent-mediated, the hydration shells reflect the asymmetry of the solute and this plays a role in the overlap of the cospheres. It was long thought that the presence of long-range electrostatic interactions and the screening effect of water exclude chiral recognition in the case of unprotected, zwitterionic α-amino acids[78]. Recently, however, it was shown in a number of microcalorimetric studies[79,80,81,82] that the zwitterionic form of the α-amino acids is actually responsible for the observation of chiral recognition in aqueous solution. These results were explained using a preferential configuration model of the hydrophilic groups[83].The carboxylate and ammonium groups of two α-amino acid molecules interact preferentially. Subsequently, the apolar α-amino acid side chains can have hydrophobic interactions in one diastereomeric pair, but not in the other, depending on whether they occupy the same half-space or not (see Scheme 1.5). This leads to differences in excess thermodynamic properties of the solution. In other words, the model accounts for the enhanced cooperation of hydrophobic interactions sustained by hydrophilic interactions[84]. The preferential configuration model, in which the nature of the polar groups determines the strength of hydrophobic interactions, was also used to interpret microcalorimetric results for aqueous solutions containing *N*-acetyl amino acids[85,86], α,ω-amino acids[87] and steric and geometric isomers of imino acids (Pro-derivatives)[88].That the zwitterionic character of the α-amino acids is the driving force for the formation of diastereomeric interactions, is shown by a similar microcalorimetric study at low pH[89], where the carboxylate groups are protonated. Chiral recognition is then lost, emphasising the importance of specific hydrophilic interactions in the molecular recognition process. The nature of the α-amino acid side chain affects the observed differences in excess enthalpies. Differences in enthalpic pairwise coefficients



Scheme 1.5 *Schematic representation of the preferential configuration model*.

between isomers of the same and isomers of opposite chirality show up most clearly for α-amino acids bearing longer alkyl chains, such as Leu, Ile and nLeu[79] (see Figure 1.2), due to more pronounced hydrophobic interactions in the LL and DD pairs. When the SWAG approach is applied to positional isomers or stereo isomers, it obviously fails. When urea is added to the aqueous medium[90], the extent of chiral recognition is influenced in the case of Leu only, indicating the inferior role of the shorter alkyl chains in the recognition process. The presence of a hydrophilic group in the alkyl chain enhances side chain-side chain hydrophobic interactions in the homochiral configuration[81]. Only in a few exceptional cases, as in the case of Phe, side chain-side chain interactions dominate the molecular recognition, *i.e*. are stronger than the hydrophilic interactions. Consequently, the preferential configurationand subsequently the chiral recognition is then lost[82]. An important conclusion from these studies is that hydrophilic interactions are the driving force for the intermolecular interaction but that the hydrophobic interactions are indispensable for chiral recognition.

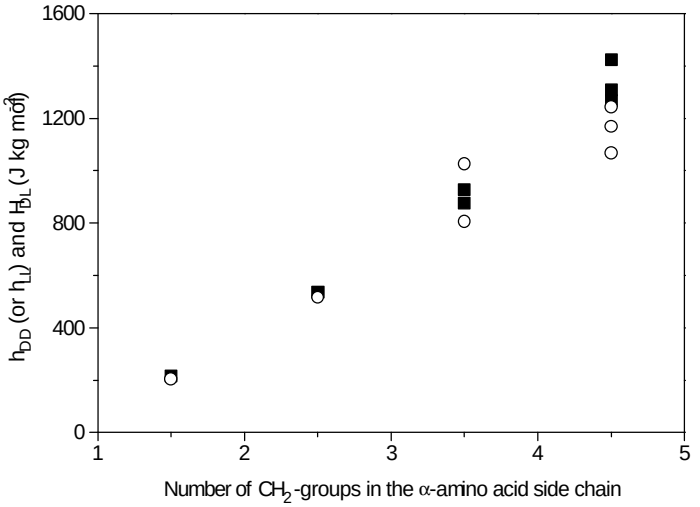


Figure 1.2 *Enthalpic pairwise interaction coefficients, h_{DD} (or h_{LL}) (■) and (h_{DL}) (O), for apolar α-amino acids.*

1.4.3.5 Other methods employed to study α-amino acid hydration

Besides the study of partial molar properties and α-amino acid-solute interactions by microcalorimetric methods, there are other ways to obtain information of α-amino acid hydration. The use of theoretical methods has already been mentioned. Dixit *et al*.[91] calculated the electrostatic contribution of zwitterionic α-amino acids to the solvation Gibbs energy. Since the largest part of the electrostatic contribution stems from the charged groups, the variable values for the 20 α-amino acids clearly indicate the effect of the side chain on the zwitterionic solvation (*i.e*. intramolecular hydration shell overlap effects). Also the solvation Gibbs energy is less favourable

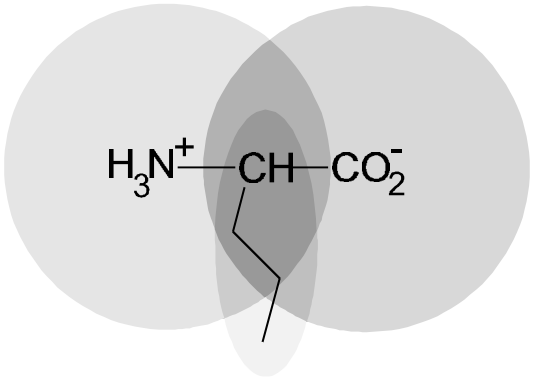
for Ile compared to Leu, emphasising the influence of the branching of the alkyl group on hydrophilic hydration. Of interest is also a recent study by Kollman *et al*.[92], in which molecular dynamics simulations of aqueous solutions containing amongst others $CH_3CO_2^-$ and $CH_3NH_3^+$ are described. It was confirmed that water structure surrounding the methylene groups is not enhanced but maintained with respect to bulk water. Water-water interactions around polar groups are sacrificed to enhance water-polar group interactions. The hydration of methyl groups is not uniform and depends on the neighbouring groups (*i.e*. failure of the additivity approach). Computational methods also predict the destructive effect of ionic hydration on hydrophobic hydration[70] in an α-amino acid.

Suzuki *et al*.[93] studied the hydrophobic hydration of α-amino acids by a microwave dielectric method, where the rotational relaxation of restrained water by the hydrophobic side chains was separated from the dielectric spectrum of the α-amino acid solution. On average, three water molecules are constrained by each carbon atom. A clear distinction between linear and branched alkyl chains (Val/nVal and Leu/nLeu) was observed. The linear alkyl chains restrained more water molecules in their rotational motion, indicating a more hydrophobic behaviour.

Neutron scattering experiments provide direct evidence for the modified water structure within the hydration shell of a hydrophobic *N*-acetylamino acid amide compared to a hydrophilic *N*-acetylamino acid[94].

NMR techniques[95,96] have also been employed for the study of α-amino acid and peptide hydration. They provide additional evidence for the occurrence of intramolecular hydration shell overlap.

1.5. Intramolecular hydration shell overlap effects



Scheme 1.6 *Pictorial representation of the overlapping hydration shells of norvaline in*

The partial molar properties of aqueous α-amino acid solutions as well as studies describing solute-solute interactions involving α-amino acids indicate that the hydrophilic and hydrophobic hydration shells of their functional groups overlap destructively, where hydrophilic hydration destroys hydrophobic hydration, and vice versa. This overlap can cause deviations from additivity of apolar group interactions, which are indeed observed when solution

aqueous solution.

data for α-amino and derivatives are analysed. The position of the apolar group in the polyfunctional molecule is important, or more specifically, its distance to hydrophilic groups seems to determine its apparent hydrophobicity. In spite of the enormous research effort in this area, the basic understanding of the role of solute-solute interactions, where the solute contains both polar and apolar regions, is far from complete. General rules are required which allow estimates of the thermodynamics which generally determine what are currently called molecular recognition progresses.

1.6 Aim of the study and survey of the contents

The main objective of the present study was to obtain quantitative information about the pairwise noncovalent solute-solute interactions in aqueous solution, involving solutes with distinct polar and apolar groups, with the main interest in the polyfunctional α-amino acids. In addition, other biologically relevant solutes have been examined. Among the noncovalent interactions, the focus is especially on the hydrophobic interactions of the apolar groups and how these are affected by the hydration of the polar groups of the solutes. To date, thermodynamic data on α-amino acid-solute interactions are mainly enthalpic. Although the enthalpy is a powerful thermodynamic quantity, it is not by itself sufficient to indicate whether the interactions are favourable or unfavourable. The impetus of the study was to obtain insight into the excess Gibbs energies of, among others, α-amino acid solutions, by which pairwise Gibbs energy interaction parameters can be obtained.

Accurate values of these parameters can be obtained by measuring kinetic solvent effects. The rates of (water-catalysed) hydrolysis reactions using UV/Vis-spectroscopy are determined in the presence of small amounts of α-amino acids, cyclic amides and alkylated ammonium bromides. Using a thermodynamic description of kinetic solvent effects (see Section 2.2), the kinetic results are expressed in pairwise Gibbs energy interaction parameters. On a more detailed level, these parameters have been subject to an analysis in terms of additivity of functional group contributions.

In Chapter 2, the experimental pathway is outlined and an overview of previous, related studies is given. Subsequently, the kinetic solvent effects of *N*-alkyl-2-pyrrolidinones and structurally related compounds on the hydrolysis reactions of two activated amides (1-benzoyl-1,2,4-triazole and 1-benzoyl-3-phenyl-1,2,4-triazole) and an activated ester (*p*-methoxyphenyl dichloroacetate) have been

studied. These solutes are model compounds for cyclic peptides and the kinetic results yield information about amide hydration, which is an important issue in the literature in view of the driving forces governing protein folding[22]. It is seen that hydrophobic interactions are attenuated by the amide hydration shell, which appears to extend to the third carbon atom of the apolar alkyl group of the solutes.

In Chapter 3, the kinetic solvent effects of alkylated ammonium bromides on the hydrolysis of 1-benzoyl-1,2,4-triazole are described. The cationic ammonium group is found again in zwitterionic α-amino acids, but in the alkylated ammonium bromides the cationic group is not accompanied by carboxylate group effects. Therefore, cationic hydration effects can be studied in an undisturbed way, which is useful in the discussion of the more complex hydration of zwitterionic α-amino acids. It is observed again that the formation of a polar hydration shell is at expense of the formation of the hydrophobic hydration shell. The cationic hydration shell, like the nonionic amide hydration shell, extends to the third carbon atom in the apolar alkyl substituents.

The kinetic solvent effects of α-amino acids and derivatives on activated amide hydrolysis reactions (including 1-benzoyl-3-phenyl-1,2,4-triazole) are described in Chapter 4. Generally, the zwitterionic character of these solutes completely overshadows hydrophobic interactions involving the apolar side chains. Hydrophobic interactions only play a role for solutes protected at the carboxylate terminus and for solutes with aromatic groups in the side chain, like for Phe. In addition, isobaric activation parameters are determined for some α-amino acids solutions, to obtain more insight into the complex interplay of noncovalent interactions involving zwitterionic α-amino acids.

The kinetic solvent effects of anionic α-amino acids (*i.e*. at high pH) and some peptides on the S_N1 hydrolysis of 2-(4-nitrophenoxy)tetrahydropyran are analysed in Chapter 5. Combined amino and carboxylate groups have a different effect on hydrophobic interactions involving the apolar side chains than the combined ammonium and carboxylate groups in zwitterionic α-amino acids. The use of a different substrate highlights different aspects of α-amino acid hydration. In Chapters 2-5, additivity of pairwise apolar group interactions is discussed.

An appendix reports preliminary investigations of interactions of zwitterionic α-amino acids (and some peptides) with vesicle bilayers, which are investigated by fluorescence depolarisation spectroscopy. An attempt was made to find a relationship between the α-amino acid side chain hydrophobicity and the extent to which the bilayer alkyl chain packing is perturbed.

In Chapter 6, the kinetic results of the different solutes used in Chapters 2-5 are compared in terms of intramolecular hydration shell overlap effects. Particular attention is paid to the noncovalent interactions of the hydrophilic groups.
Finally, summaries are given in English and in Dutch.

Most of the work described in this thesis has been published or will be published in the near future[97].

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CHAPTER 2

Effects of Polar Nonionic Group Hydration on Noncovalent Interactions

2.1 Introduction. Kinetic solvent effects

The reactivity of molecules in solution is largely dictated by the solvent. Sometimes a change in solvent can lead[1] to rate effects in the order of 10^9. Solvent effects have also been reported for other chemical phenomena, such as chemical equilibria, spectroscopic parameters, isomerisations[2], aggregation[3], ionisation[4] and conformation[5], but the effects on chemical reactions have received most of the attention.

Many chemical reactions are performed in mixtures of solvents. Kinetic solvent effects on organic reactions in mixed aqueous solvents are an important topic of study in physical organic chemistry. Patterns of organic reactivity in these solvents are particularly interesting[6,7]. Organic reactivity in aqueous solution is receiving increasing attention, due to the need of environmentally friendly substitutes for toxic organic solvents[8]. Organic cosolvents or cosolutes are often added to increase the solubility of the reactant(s). (The term cosolvent is used when the component in the solvent mixture is present in a comparable amount with the reference solvent. The term cosolute is used for dilute mixed solvents. In this thesis the term cosolute is used throughout.)

Frequently, (kinetic) solvent effects have been explained qualitatively in terms of macroscopic properties of the solvent which reflect the polarity of the solvent[9,10,11]. However, for mixed (aqueous) solvents these methods are not adequate. A major drawback is that the solvent is described as a continuum and, consequently, changes in the local structure of the solvent in the solvation shells of the reactants during the activation process are neglected. Therefore, a new model was required to quantitatively analyse kinetic solvent effects in these mixed (aqueous) solvents.

2.2 Quantitative analysis of kinetic solvent effects in dilute aqueous solutions in terms of pairwise Gibbs energy interaction parameters

The kinetics of reactions in aqueous solutions are particularly interesting because the activation parameters are strongly affected by changes in the structural features of the solvent cosphere around the reactant during the activation process[12,13]. This sensitivity is mainly because water is such a highly structured solvent. Changes in solvent composition, for example by adding small amounts of cosolutes, affect the hydration characteristics of both reactant and activated complex due to interactions with the cosolute via overlap of hydration cospheres[14]. The way in which the reactivity of the reactant responds reveals the characteristics of the interactions between the medium and the reactant and activated complex and the response indirectly provides information about the cosolute hydration.

Transition state theory provides the basis of understanding kinetic medium effects. The interacting cosolutes can decrease or increase the chemical potentials of the initial state and the activated complex, depending on whether the interactions are favourable (stabilising) or not favourable (destabilising), and consequently affect the Gibbs energy of activation.

Since the main interest is in the pairwise Gibbs energies of interaction between the solutes, experimental rate constants need to be converted into thermodynamic parameters describing these interactions. About a decade ago, a theory was developed with which kinetic medium effects of solvolysis reactions can be analysed quantitatively in terms of thermodynamic interaction parameters[15,16]. The Gibbs energy of interaction stems from the nonideal part (the excess Gibbs energy (G^E)) of the total solution which is determined by the chemical potentials of the solutes and this nonideal part can be ascribed completely to solute-solute interactions. Usually, G^E is expressed by a molality expansion, using virial coefficients. These coefficients are given physical significance by the theory of McMillan and Mayer[17]. In sufficiently dilute solutions G^E is determined by pairwise solute-solute interactions only. The thermodynamics were then linked with kinetics through transition state theory, yielding the following equation for a water-catalysed hydrolysis reaction[15,16]:

$$ln\frac{k(m_c)}{k_0(m_c=0)} = \frac{2}{RTm_0^2}(g_{c\text{-}IS} - g_{c\text{-}AC})m_c - nfM_w m_c$$

where k is the pseudo-first-order rate constant for reaction in the aqueous solution containing the cosolute c, k_0 the pseudo-first-order rate constant for reaction in pure water, R the gas constant, T the temperature, m_0 the standard state: 1 mol kg^{-1}, m_c the molality of the cosolute, n the number of water molecules involved in the transition state of the hydrolysis reaction (n = 2 in the hydrolysis reactions studied in

this thesis, with the exception of the hydrolysis reaction described in Chapter 5), M_w the molar mass of water and ϕ the practical osmotic coefficient (which equals unity in dilute aqueous solution). The term between brackets is referred to as the G(c) value, the pairwise Gibbs energy interaction parameter, which is the difference in pairwise interactions of the cosolute (c) with the initial state (IS) and the activated complex (AC) in the transition state.

The second half of the equation reflects the effect of the cosolute on the reactivity of water, since water is solvent as well as reactant.

Thus, G(c) represents the overall effect of the cosolute on the Gibbs energy of activation for the hydrolytic process. The G(c) is obtained from the slope of a plot of ln (k/k_0) versus the molality of the cosolute.

As stated at the beginning of this section, it is assumed that the solutes interact via their hydration spheres; solvent spheres around the solute which have structural and dynamical properties different from those of the bulk solvent. These solvent spheres can interact in such a way that they are either compatible, leading to an attractive (stabilising), or incompatible, leading to a repulsive (destabilising) interaction when they overlap.

Direct interactions between the solutes should not be completely neglected, particularly in cases where dehydration of the solutes costs little Gibbs energy (*i.e*. where solute↔water interactions are relatively weak). However, all solutes investigated in this thesis contain hydrophilic moieties which interact strongly with water, primarily via hydrogen bonding. The dehydration processes of these solutes have a high energy barrier. Hence, whenever noncovalent solute interactions are mentioned in this thesis, these are presumed to take place largely via hydration sphere overlap processes, through at least one layer of intervening water molecules, and not via direct interactions.

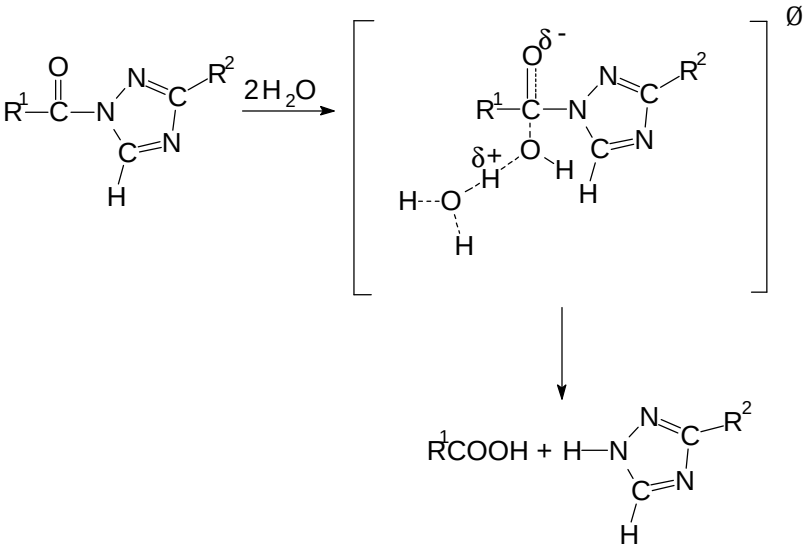
A more detailed use of the theory for quantitative analysis of kinetic solvent effects allows the evaluation of the G(c) parameters in terms of pairwise group energy interaction parameters: the SWAG (Savage and Wood Additivity of Groups[18]) approach (Chapter 1).

2.3 Kinetic solvent effects on the neutral hydrolysis of 1-acyl-(3-substituted)-1,2,4-triazoles: an overview

The quantitative treatment of rate constants, as described in Section 2.2, has been applied to kinetic solvent effects of several hydrolysis reactions in dilute aqueous media, including the neutral (water-catalysed) hydrolyses of perchlorates[19],

activated esters[15] and activated amides[16,24,25,27-33]. In particular, the kinetics of the neutral hydrolyses of 1-acyl-3-substituted-1,2,4-triazoles have been investigated in the presence of numerous cosolutes. The pseudo-first-order hydrolysis reaction proceeds via a dipolar activated complex in which two water molecules are involved, with three protons 'in flight'[20] (see Scheme 2.1). The difference in hydrophobicity between the initial state and the activated complex in the transition state is responsible for the marked changes in rate constants which are observed when hydrophobic cosolutes are added.

Scheme 2.1 *Reaction mechanism for the water-catalysed hydrolysis of 1-acyl-3-substituted-1,2,4-triazoles. For 1-benzoyl-1,2,4-triazole (**BT**) $R^1=C_6H_5$ and $R^2=H$, for 1-benzoyl-3-phenyl-1,2,4,-triazole (**BPhT**) $R^1=R^2=C_6H_5$.*



The reasons for choosing this type of hydrolysis reaction were fourfold:

1) Water plays a crucial role in the formation of the activated complex, hence changes in hydration of the interacting solutes are likely to affect the reaction rate.
2) The hydrophobicity of the reactant can be fine-tuned through variation of the substituents.
3) The reactions are rather insensitive towards acid catalysis, in particular for 1-benzoyl-1,2,4-triazole[21], which allows the measurement of rate constants for the water-catalysed hydrolysis in a relatively broad pH range (pH 3-5).
4) There is no need for perturbing the aqueous solution by the presence of a buffer in the relevant pH region.

In Table 2.1, the kinetic solvent effects on the hydrolyses of the activated amides 1-benzoyl-1,2,4-triazole (**BT**) and 1-benzoyl-3-phenyl-1,2,4-triazole (**BPhT**) and the activated ester *p*-methoxyphenyl dichloroacetate (of which the hydrolysis proceeds

via a similar reaction mechanism to that of the amide hydrolysis of Scheme 2.1.), which have been analysed in terms of pairwise Gibbs energy (group) interaction parameters, are summarised, including the studies described in this thesis. These solvent effects will be discussed briefly. All cosolutes retarded the hydrolysis reactions in dilute aqueous solution (with exception of the α-amino acids; see Chapter 4). The retardations have been shown to be governed by hydrophobic interactions between the cosolute and the reactant causing a stabilisation of the initial state, which is much larger than that of the transition state[22]. The retardation reflects the loss of hydrophobicity of the reactant during the activation process as well as the overall hydrophobic character of the added cosolutes.

Table 2.1 *Kinetic solvent effect studies on the hydrolyses of* ***BT****,* ***BPhT*** *and p-methoxyphenyl dichloroacetate (****MPDA****) in dilute aqueous media, which have been analysed in terms of pairwise Gibbs energy interaction parameters.*

	References	
Cosolvent	**Activated amides**	**Activated ester**
mono-, di- and polyhydric alcohols	16, 23	
(alkyl substituted) urea(s)	24	15, 24
carboxamides	26	26,27
sulfonamides/sulfones/sulfoxides	24	24, 25
carbohydrates	26, 27	
N-alkyl-2-pyrrolidinones	28 (this chapter)	28
sodium *n*-alkylsulfates	29	
n-alkylated ammonium bromides	30 (chapter 3)	
α-amino acids	31, 32 (chapter 4)	

For some solutes the pairwise Gibbs energy interaction parameters could be described in terms of pairwise *group* interactions in dilute aqueous solutions (using the SWAG-approach), but for many cosolutes the G(c) values could not be analysed using this approach.

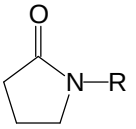
In the case of monohydric alcohols as cosolutes, G(c) values could be analysed satisfactorily in terms of the SWAG approach of pairwise functional group interactions between cosolute and hydrolytic probe and estimates for G(CH) and G(OH) were obtained. However, when diols and polyols were considered, the contribution of Gibbs energy interactions of the CH- and OH-groups to the overall medium effects altered significantly, depending on the location of the hydroxyl groups in the cosolute molecule. This dependence was caused primarily by OH-OH interactions which appeared to take place over a distance of 3 intervening carbon

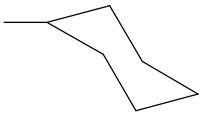
atoms. Clearly, an analysis in terms of additivity of functional group parameters was impossible. After this application of the theory to alcohols, the investigation was extended to other, more complex, cosolutes. However, generally, those studies have not been very successful in terms of additivity of pairwise functional group interactions either. The (alkylated) ureas did show additivity of functional group interactions in the interaction with the kinetic probes as far as the limited amount of data suggested. So did the carboxamides in interactions with the activated ester, but additivity of functional groups was not observed for interactions of carboxamides with the activated amide, presumably due to specific amide-amide interactions. Since the additivity theory does not allow for specificity in interactions for different structural isomers and stereoisomers, it was anticipated that these would bring about similar rate effects. However, for the isomeric carboxamides and, more clearly, for carbohydrates as cosolutes, this is not the case. According to these kinetic data sets, additivity of pairwise functional group interactions is more the exception than the rule. Even the data for the alcohols show, upon re-evaluation, deviations from additivity, but this was not recognised as such originally[16]. Actually, the group interaction parameters depend strongly on the other functional groups in the solute, their relative position and their stereochemistry. To obtain more insight into the extent of destructive intramolecular hydration shell overlap and their effects on intermolecular interactions, the kinetic studies were extended to solutes with distinct hydrophilic groups. Some interesting features have been elucidated using sodium *n*-alkylsulfates as cosolutes, the first solutes containing an anionic group. They possess an extensively hydrated polar group (mainly through strong hydrogen bonding interactions) and the apparent hydrophobicity of methylene groups up to the third carbon atom removed from the charged sulfate group is reduced tremendously. In this chapter, we report the kinetic medium effects of *N*-alkyl-2-pyrrolidinones, highly water-soluble solutes of which the hydrophobicity can be systematically varied and which also contain an amide group.

After the investigation of anionic cosolutes, the effects of the cationic (tetra)alkylammonium bromides also provide a valuable contribution to the understanding of ionic hydration and their effects will be discussed in the next chapter. α-Amino acids are of particular interest. Their zwitterionic character can be expected to have far-reaching consequences for intermolecular interactions in aqueous solution. The solvent effects of these solutes on hydrolysis reactions in dilute aqueous solution are described in Chapter 4 and Chapter 5.

2.4 Kinetic solvent effects of *N*-alkyl-2-pyrrolidinones on three water-catalysed hydrolysis reactions

N-Alkyl-2-pyrrolidinones, cyclic amides with alkyl substituents at the nitrogen atom, owe their good solubility in water to the amide functionality which causes *N*-cyclohexyl-2-pyrrolidinone even to be miscible with water in all proportions. Therefore, they are suitable solutes for studying hydrophobic interactions in aqueous solution. It is of interest to study the effects of this class of cosolutes on activated amide hydrolyses because the data are expected to provide more insight into noncovalent amide-amide interactions in aqueous solution, interactions of which the energetics are still not completely understood[33] and which are important in protein folding processes. In addition a comparison with the acyclic amides can be drawn[24,25]. The five substituted pyrrolidinones that we have studied are listed in Scheme 2.2. *N*-Cyclohexyl-2-pyrrolidinone is in fact not part of this series of homologues, but is interesting in view of its unlimited solubility in water and furthermore can provide information about differences in hydrophobicity of cyclic and acyclic alkyl substituents The main objectives of the study are to investigate whether the solvent effects caused by these cosolutes can be quantitatively described in terms of pairwise Gibbs energy interaction parameters between cosolute and kinetic probe. Secondly, the additivity of apolar group interactions will be investigated in terms of interactions between the hydration shells of the different functional groups. In view of the remarkable results obtained for the acyclic amides with regard to additivity of functional groups (*i.e*. additivity of groups was observed in case of the hydrolysis of *p*-methoxyphenyl dichloroacetate (**MPDA**), but not for 1-benzoyl-3-phenyl-1,2,4-triazole (**BPhT**)[24]), solvent effects of the *N*-alkyl-2-pyrrolidinones have also been determined for the hydrolysis of **BPhT** and **MPDA**, in order to investigate whether a similar pattern is found for these solutes. Also 1-benzoyl-1,2,4-triazole (**BT**) has been used as a kinetic probe, being less hydrophobic than **BPhT** and therefore useful for studying the solvent effects in terms of hydrophobic interactions.



—R	Abbr.
—CH_3	NMP
—CH_2CH_3	NEP
—$CH(CH_3)_2$	NiPP
—$CH_2CH_2CH_2CH_3$	NnBP
	NCHP

Scheme 2.2 *N-Alkyl-2-pyrrolidinones used in the present kinetic study and their abbreviations used throughout the chapter.*

Solvent effects of the *N*-alkyl-2-pyrrolidinones have been measured at different molalities up to several molal concentrations of cosolute at 25°C and pH 4. As an example, the solvent effects of the series in Scheme 2.2 on the hydrolysis of **BPhT** are shown in Figure 2.1 up to 1 molal concentration. Generally, linear relationships between $\ln(k/k_0)$ and the molality of the cosolute have been obtained up to approximately 0.75 molal[a], indicating pairwise interactions between cosolute and kinetic probe. From the slopes of the lines in Figure 2.1, the G(c) values are obtained and are tabulated in Table 2.2, together with the G(c) values obtained for the hydrolyses of **BT** and **MPDA**. To illustrate the dependence of G(c) on the structure of the cosolute, G(c) values have also been plotted versus the number of CH_2-groups in the alkyl substituent (Figure 2.2). In all cases, the *N*-alkyl-2-pyrrolidinones caused a retardation of the hydrolysis reactions, expressed by negative G(c) values. These retardations can largely be attributed to favourable interactions between apolar moieties in the cosolute and the reactant molecule, *i.e*. an initial state stabilisation due to hydrophobic interactions.

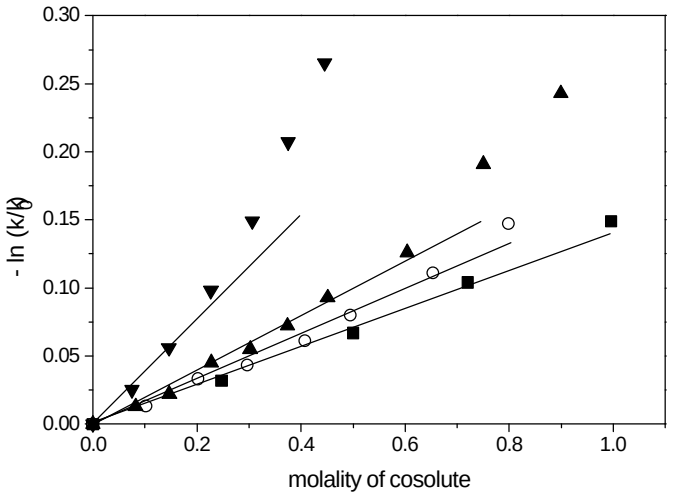


Figure 2.1 *Solvent effects on the hydrolysis of* ***BPhT*** *in the presence of NMP (■), NEP (O), NiPP (▲), NnBP (▼).*

Table 2.2 *G(c) values[a] (J kg mol^{-2}) for the different cosolute-probe combinations.*

	probe		
cosolute	**MPDA**	**BPhT**	**BT**
NMP	-925 (10)	-133 (4)	-292 (10)
NEP	-1176 (18)	-157 (10)	-354 (11)
NiPP	-1407 (10)	-206 (15)	-354 (4)
NnBP	-1989 (16)	-467 (21)	-600 (15)
NCHP	-2980 (23)	-1000 (40)	-600 (41)

[a]Errors in parenthesis

[a]This is with the exception of NCHP as a cosolute. Solvent effects of NCHP on the three hydrolysis reactions show linear behaviour up to 0.2-0.3 molal. Above these concentrations higher-order interactions come into play. Collection of kinetic data up to 3 molal yielded S-shaped curves, which could be perfectly analysed with the Menger-Portnoy model[34] for micellar catalysis, indicating the formation of NCHP aggregates. This was confirmed by fluorescence spectrophotometric measurements using pyrene as a probe which is sensitive to the solvent microenvironment[35].

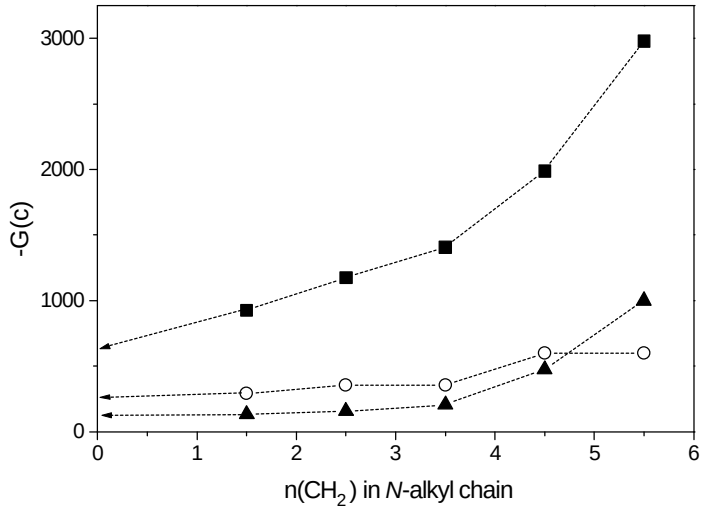


Figure 2.2 *G(c) values (J kg mol^{-2}) versus the number of CH_2-units in the N-alkyl chain of N-alkyl-2-pyrrolidinones for the kinetic probes* ***MPDA*** *(■),* ***BPhT*** *(▲) and* ***BT*** *(O).*

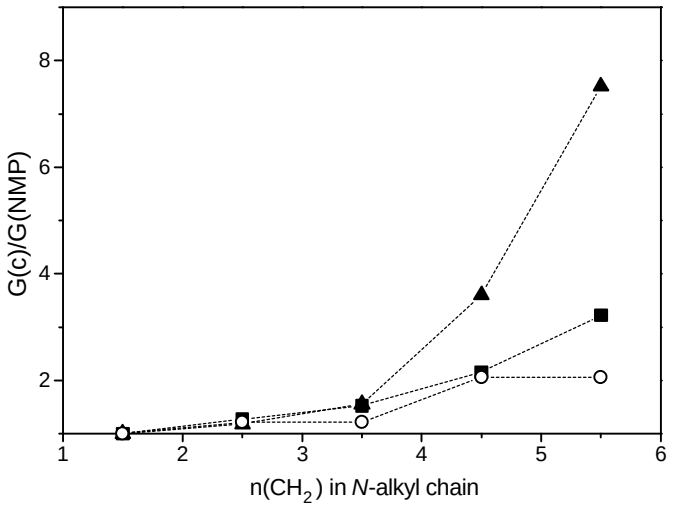
It is remarkable that the ester hydrolysis is retarded to a much larger extent than the hydrolyses of both **BT** and **BPhT**. In other words, the ester hydrolysis seems to be more sensitive towards solvent effects than the amide hydrolyses. This is in accordance with earlier findings[24]. This increase in activation energy for the ester hydrolysis is unlikely to be governed by increased stabilisation of the initial state, because the ester probe is less hydrophobic than the amide probes. However, it is possible that the initial state is stabilised by other noncovalent interactions or that the larger retardations have to be attributed primarily to a transition state effect. Noncovalent interactions other than hydrophobic interactions that may be operative in the initial state are N-H···O=C hydrogen bond interactions. These are of similar strength for amide-amide and ester-amide interactions according to a theoretical investigation of hydrogen bonding between formamide and *Z*-methyl acetate[36], but they may depend on the geometric requirements of interactions between the solutes. Apparently the ester can accommodate an intermolecular hydrogen bond more easily than the more crowded amide, which would explain additional stabilisation. However, it has been suggested that amides form stronger H-bonds with water than with other amides[37,38], *i.e*. the solute-solvent interactions dominate the solute-solute interactions and the same is anticipated for the ester functionality. In that case, the larger solvent effects for the ester hydrolysis must find their origin in a transition state effect, *i.e*., the transition state of the amide hydrolysis is more stabilised than the transition state of the ester hydrolysis.

The transition state of the ester hydrolysis is less polar than that of the amide hydrolyses (*i.e*. the difference in polarity between IS and TS is smaller for the ester hydrolysis, as is reflected by a larger k_0). There is an increase in polarity of the solvent when the rather polar *N*-alkyl-2-pyrrolidinones are added to the medium, which would explain the larger stabilising effect on the transition state of the triazole hydrolysis due to polar interactions.

In conclusion, an interpretation of the results in Table 2.2 solely in terms of hydrophobic effects is most likely misleading. **MPDA** *appears* to be able to interact more strongly with the cosolutes via hydrophobic interactions than **BT** and **BPhT**, but this is in fact not true. When the data in Table 2.2 are treated in a way where the retardations are viewed with the NMP retardations as a reference, rather than the rate of hydrolysis in pure water, another pattern emerges. This pattern is shown in Table 2.3 and visualised in Figure 2.3.

Table 2.3 *G(c) values for the different cosolute-probe combinations relative to the G(c) of NMP.*

	probe		
cosolute	**MPDA**	**BPhT**	**BT**
NMP	1	1	1
NEP	1.27	1.18	1.21
NiPP	1.52	1.55	1.21
NnBP	2.15	3.60	2.05
NCHP	3.22	7.52	2.05



In terms of this approach, the different probes are more comparable, since the differences in transition state stabilisation are ruled out and therefore initial state effects (hydrophobic effects) might show up more clearly. This seems to be indeed the case. As anticipated, the hydrolysis of the more hydrophobic probe (**BPhT**) is then relatively the most sensitive to the hydrophobicity of the cosolute.

Figure 2.3 *G(c)/G(NMP) versus the number of CH_2-units in the N-alkyl chain of N-alkyl-2-pyrrolidinones for the hydrolysis of* ***MPDA*** *(■),* ***BPhT*** *(▲) and* ***BT*** *(O).*

Thus, it seems that although the *absolute* values of the medium effects are not solely governed by hydrophobic interactions, relative values reveal that these type of noncovalent interactions play an important role in the recognition process between the cosolutes and the kinetic probes.

The next topic of interest is whether the medium effects, as expressed in G(c) values, can be analysed in terms of additivity of pairwise group interactions, as was suggested by Savage and Wood[39], *i.e*. does each CH_2-unit in the cosolute molecule, irrespective of its position, yield a constant increment to the medium effect? Figure 2.2 shows that this is not the case throughout the whole series of *N*-alkyl-2-pyrrolidinones, since there is no linear relationship between G(c) and the number of CH_2-units. It seems that the longer the alkyl chain, the larger the contribution of a CH_2-unit towards the medium effect. This pattern is particularly pronounced for solutes with larger apolar groups than NiPP. It appears that for the shorter alkyl chains additivity of CH_2-interactions may apply. In Figure 2.4, the G(c) values are plotted against n(CH_2) but now on a larger scale and for NMP, NEP and NiPP only. Additivity is reasonably good, with slopes representing the G(CH_2) which are displayed in Table 2.4. Thus there is additivity up to 3.5 CH_2-units (3 carbon atoms attached to nitrogen) but the longer alkyl chains deviate from this additivity pattern. The G(CH_2) value for **MPDA** is more negative than that obtained for a series of acyclic primary, secondary and tertiary amides for the same kinetic probe[24]. On the contrary, for the hydrolysis of **BPhT** a more negative value is found for the cyclic amides. This is not easily explainable in terms of pairwise group interactions. There are too many noncovalent interactions playing a role in both initial state and

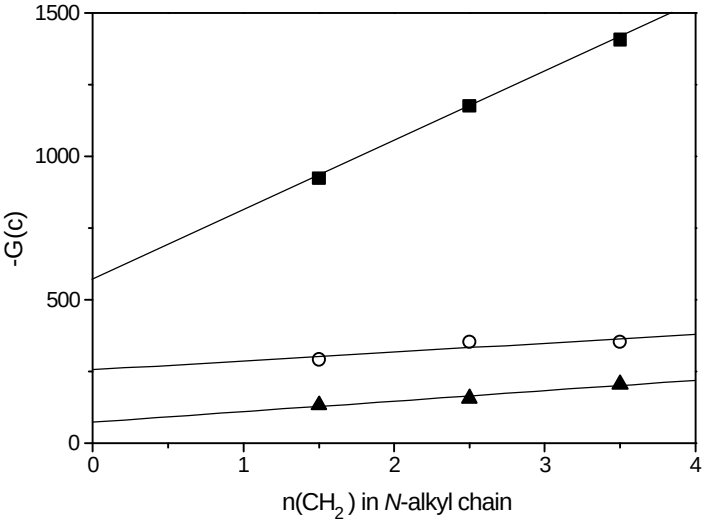


Figure 2.4 *Enlargement of Fig.2.2 (same symbols apply) to show additive behaviour in short-chained N-alkyl-pyrrolidinones.*

Table 2.4 *$G(CH_2)$ values in J kg mol^{-2} for several cosolute-probe combinations.*

	cosolutes		
probe	cyclic amides	acyclic amides[24]	alcohols[21,23]
MPDA	-241	-142	n.d.[a]
BPhT	-37	-51	-136
BT	-31	n.d.[a]	-90

[a]Not determined.

transition state which are difficult to distinguish from each other. Moreover, since the *N*-alkyl-2-pyrrolidinones are tertiary amides, a comparison might not be fully justified. The $G(CH_2)$ values for the cyclic amides are about one third of the values obtained for short chain alcohols for the hydrolyses of **BPhT** and **BT**. This seems to indicate that the amide group is more extensively hydrated than the alcohol functionality, therefore diminishing the interactions of the CH_2-units with the kinetic probe more. Or, differently formulated, the hydration shells of the polar and apolar parts of the cosolutes are more incompatible in the case of the cyclic amides.

The intercepts in Figure 2.4 represent the contribution of the pyrrolidinone unit (p) towards the medium effect. The G(p) values are -567 (**MPDA**), -74 (**BPhT**) and -25 (**BT**) J kg mol^{-2}. The fact that these values are different for the different kinetic probes indicates again that there are specific cosolute-probe interactions operative, most likely in the initial as well as in the transition state. An extrapolation towards a contribution for the amide (CONH) unit only (*i.e.* $n(CH_2)_{total} = 0$) is not so straightforward either, since this would change the G(c) due to the contribution of three CH_2-units located in a ring system. It was shown previously that CH_2-units in a ring can either reduce the apparent hydrophobicity[16] or increase the apparent hydrophobicity[24] relative to CH_2 units which are not joined in a ring system. In addition, it has to be kept in mind that a modified G(CONH) would be obtained in this case; its solvent effect is influenced by the attached alkyl groups. A rough estimate, however, would imply a positive contribution towards G(c) for the amide functionality. This is not in agreement with previous findings, which suggest a negative contribution[24].

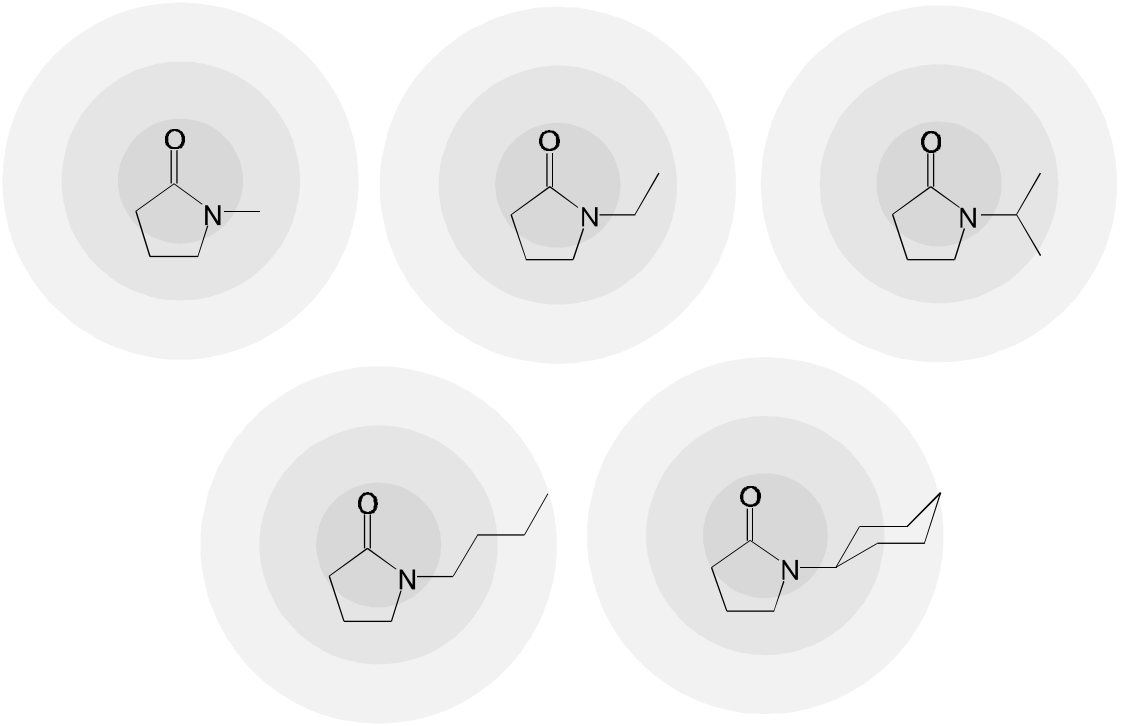
The above discussion is based on the additivity which is observed for the short-chained *N*-alkyl-2-pyrrolidinones. Upon extending the chain to *n*-butyl and *cyclo*-hexyl, deviation from additivity develops. These cosolutes cause a much larger solvent effect than is expected when the observed additivity for the short-chained solutes is extrapolated. In this thesis it will be seen that a similar pattern is observed for other cosolutes bearing hydrophilic and hydrophobic groups[40].

The explanation for this phenomenon is that the smaller alkyl substituents are entirely located within the part of the hydration sphere of the amide functionality where the hydration water is strongly directed by hydrogen bonding with the amide group. This prevents the development of hydrophobic hydration shells for these alkyl moieties which are incompatible with the hydrophilic amide hydration shell. Consequently, their availability to interact with hydrophobic groups in the substrate via hydrophobic interactions is reduced. The effect of the cosolute amide group on the hydrogen-bonding interactions are particularly sensed in the first hydration shell. Since a break in additivity is observed after 3 CH_2-units, it seems that this intramolecular hydration shell overlap stretches over one layer of water molecules in the hydration shell.

Outside the influencing effect of the amide hydration sphere CH_2-group additivity is likely to occur as well, but with a more negative value for $G(CH_2)$, since these CH_2-groups are less hindered in their interactions with the kinetic probe. Unfortunately, insufficient data are available to ascertain this view.

At first sight it is rather surprising that additivity is found for NMP, NEP and NiPP. A gradually decreasing influence of the amide functionality would be expected. But when elaborating on the assumption that the part of the amide hydration shell which affects the hydrophobic hydration of attached alkyl groups contains only one layer of water molecules, the Me, Et and *i*-Pr groups are indeed situated in this layer because the effective diameter of a water molecule[41] is 2.75 Å and the distance between N and β-C is 2.39 Å. This assumption is supported by calculations[42] on the interaction between >CH– and >NH in water which spans 3 Å.

Therefore, deviation from additivity is likely to occur already for *N*-(*n*-propyl)-2-pyrrolidinone, which contains a γ-C atom (not measured), and it shows up very clearly for NnBP. For these cosolutes, the outer CH_2-moieties are much less hampered in their hydrophobic interactions with the kinetic probes, *i.e* for the kinetic probe they have larger apparent hydrophobicities. NCHP has one more CH_2-moiety

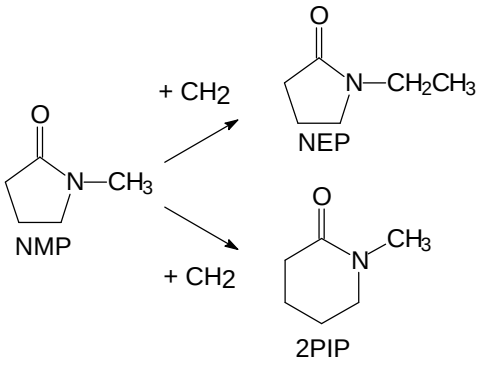


Scheme 2.4 *N-alkyl-2-pyrrolidinones with imaginary concentric spheres representing amide hydration layers of diminishing incompatibility with the apolar hydration layers. The alkyl substituents of NMP, NEP and NiPP are all situated in the same hydration layer, so are those of NnBP and NCHP.*

than NnBP, but they have similar distances between the N-atom and the most remote C-atom. This is illustrated in Scheme 2.4. For both the **MPDA** and **BPhT** hydrolyses, $|G(c)|$ doubles with an enormous decrease of 1000 and 500 J kg mol^{-2}, respectively, but interestingly for **BT** there is no difference in the G(c) value for NnBP and NCHP. In this case interactions do not seem to be of a hydrophobic nature but instead may be purely determined by effects caused by interactions involving the amide functionality which are modified by the size of the substituent.

2.5 Kinetic effects of related compounds

2.5.1 Effect of adding a CH_2-moiety either to the *N*-alkyl chain or to the pyrrolidinone ring



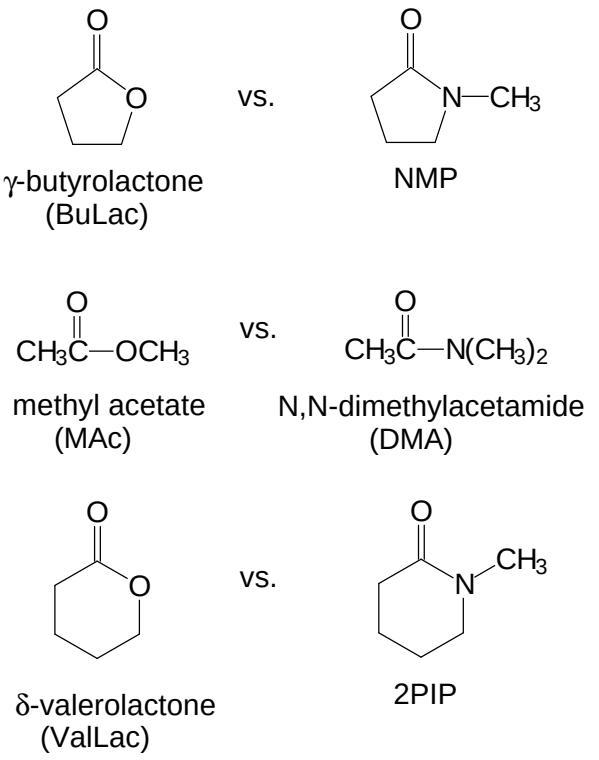
Scheme 2.5 *Addition of a CH_2-moiety to respectively the N-alkyl chain and the ring system of NMP.*

A few words have already been written on the difference in hydrophobicity between cyclic and linear alkyl chains. To give this discussion further depth, *N*-methyl-2-piperidone (2PIP), a six-membered cyclic amide has been studied. The results can be compared to the data already available for NMP and NEP (Scheme 2.5). In case of perfect pairwise group additivity, NEP and 2PIP should have the same solvent effect. G(c) values have only been obtained for the hydrolysis of **MPDA**. Due to overlap of the UV absorption spectra, medium effects on the hydrolysis of **BPhT** and **BT** could not be determined.

The G(c) value for 2PIP for the hydrolysis of MPDA is -1095 J kg mol^{-2}. Recall from Table 2.2 that the G(c) values for NMP and NEP are -925 and -1176 J kg mol^{-2}, respectively. In other words, a CH_2-moiety added to the cyclic ring contributes -170 J kg mol^{-2} to the medium effect, while a CH_2-moiety in the *N*-alkyl chain contributes -251 J kg mol^{-2}. The obvious explanation for the lower apparent hydrophobicity of the former is the reduced hydrophobic surface area, because the part of the apolar surface inside the ring is not exposed to the solvent. Therefore, interactions of that part of the molecule with (the hydration shell of) the kinetic probe do not take place. However, the conformational differences of a five- and six-membered ring may have an impact on their hydrophobicity. This would be particularly important when there is a preferential site of interaction with the kinetic probe.

2.5.2 Changing the functional group: ester versus amide functionality

In Section 2.4, it was shown that the hydrophobic effect is not the only effect determining the medium effects of *N*-alkyl-2-pyrolidinones on the hydrolysis reactions of **MPDA**, **BPhT** and **BT**. Therefore, it would be interesting to study variation in the polar (hydrophilic) functional group of the molecule. Since the investigated reactions involve the hydrolysis of an ester and two amides, it would be particularly relevant to study the effects of ester cosolutes of similar structure as the *N*-alkyl-2-pyrrolidinones. In this way information about amide-amide, amide-ester and ester-ester interactions can be obtained, providing a more complete picture of the interplay of the several noncovalent interactions governing the medium effect. In view of this discussion, the kinetic results pertinent to the structurally related esters and amides shown in Scheme 2.6 have been compared. The obtained G(c) values are compiled in Table 2.5. For a valuable comparison, the results for the ester/amide cosolute pairs shown above are also given relative to the G(c) values of the amide cosolutes in Table 2.6. From Table 2.6, it appears that the Gibbs energies of pairwise interactions of **MPDA** are rather similar for both amide and ester cosolutes, whereas for **BPhT** and **BT**, pairwise Gibbs energy interaction parameters for ester cosolutes are, respectively, 3.5 and 1.4 times more negative than those for the amide cosolutes.



Scheme 2.6 *N-alkyl-2-pyrrolidinones and structurally similar ester cosolutes.*

Clearly, the ester probe does not 'distinguish' between ester or amide solutes, whereas the amide probes do. This pattern is illustrated in Figure 2.5, where the solvent effects caused by the amide cosolutes have been plotted versus those caused by ester cosolutes. Particularly the data points for **BPhT** seem to deviate

Table 2.5 *G(c) values[a] (J kg mol^{-2}) for the three pairs of ester/amide cosolutes for the hydrolysis of **MPDA**, **BPhT** and **BT**.*

	probe		
cosolute	**MPDA**	**BPhT**	**BT**
BuLac	-836 (10)	-435 (9)	-395 (11)

NMP	-925 (10)	-133 (4)	-292 (10)
MAc	-857 (15)	-498 (13)	-408 (14)
DMA	-841 (13)	-139 (15)	-301 (14)
ValLac	-964 (18)	n.d.[b]	n.d.
2PIP	-1095 (12)	n.d.	n.d.

[a]Errors in parenthesis [b]Not possible to determine.

Table 2.6 *G(c) values (J kg mol^{-2}) for each ester/amide cosolute combination relative to the amide cosolute*.

	probe		
cosolute	**MPDA**	**BPhT**	**BT**
NMP:BuLac	1 : 0.9	1 : 3.3	1 : 1.4
DMA:MAc	1 : 1	1 : 3.6	1 : 1.4
2PIP:ValLac	1 : 0.9	n.d.[a]	n.d.

[a]Not possible to determine.

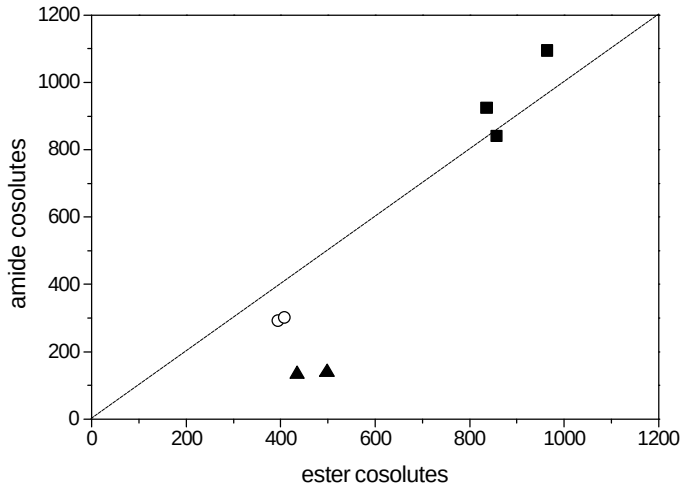


Figure 2.5 *-G(c) values of amide cosolutes plotted versus the -G(c) values of ester cosolutes for the hydrolysis of* ***MPDA*** *(■),* ***BPhT*** *(▲) and* ***BT*** *(O). Dotted line represents equal solvent effects for both amide and ester cosolutes (G(c) values in J kg mol^{-2}).*

from equal cosolute effects. How can these results be explained? The differences in retardation caused by amide and ester cosolutes for the hydrolysis of **BPhT** and **BT** do not seem to dominate the hydrophobicity of the cosolute. In terms of number of CH_2-moieties, the amide cosolutes are more hydrophobic than the ester cosolutes, since they contain an additional CH_3-group. On the other hand, the hydration of the functional group can influence the hydrophobicity of the apolar groups, so it might be erroneous to assume that the amide cosolutes are more hydrophobic. Probably their hydrophobicities are similar and thus do not explain the observed effects. Previously it was emphasised that amides are rather polar compounds. In fact they are more polar than esters. For example, IR wavenumbers for C=O stretching vibrations are 1775 cm^{-1} and 1700 cm^{-1} for ValLac and NMP, respectively[43]. Also, measurements regarding the strength of H-bond interactions

with phenol as an H-bond donor show that amides are more polar than esters (Table 2.7).

Table 2.7 *Reduction of the OH stretching frequency of phenol for some cosolutes when they become hydrogen bonded to phenol.*

Cosolute	$\Delta\nu_{OH}$ (cm^{-1})[a]
DMA	342
NMP	330
Mac	170
BuLac	190
ValLac	185

[a]Taken from ref.44.

Since the amide cosolutes are better H-bond acceptors, they can stabilise the polarised transition state of the hydrolysis reactions to a greater extent than the ester cosolutes can. This larger stabilisation of the transition state would explain the smaller kinetic medium effects caused by the amide cosolutes for the hydrolysis of **BT** and **BPhT**, but not by **MPDA**. On the whole, the G(c) values for the ester and amide cosolutes on the hydrolysis of **MPDA** are remarkably similar (see Table 2.5). Despite the fact that the **MPDA** hydrolysis is the most sensitive to changes in the medium, it is obviously not very sensitive to changes in functional groups (*i.e*. amide vs. ester functionality) or to the exact structure of the cosolute. This observation could lead to the conclusion that medium effects on the hydrolysis of **MPDA** do reflect the hydrophobicity of the cosolute better than **BT** and **BPhT**. However, a clear correlation between the G(c) and n(CH_2) does not appear to exist (see Figure 2.6). Presumably, the insensitivity to the polar group is fortuitous and caused by a number of counteracting contributions to the Gibbs energy of interaction, leading to comparable retardations for ester and amide cosolutes.

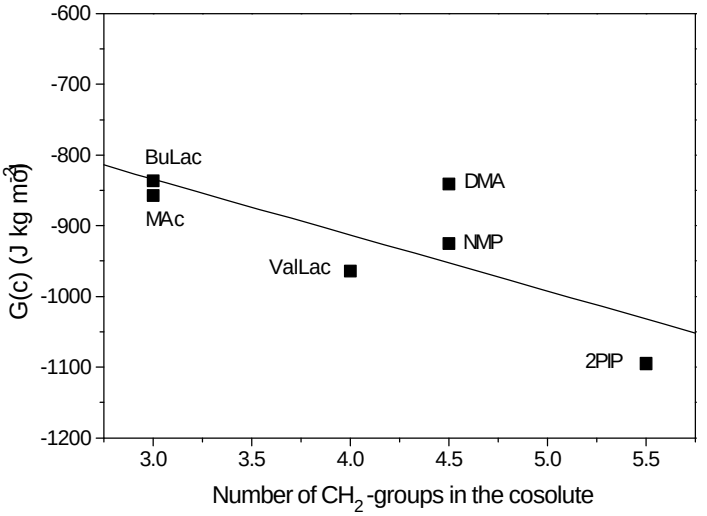
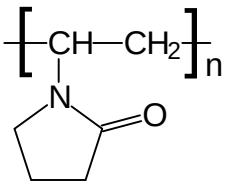


Figure 2.6 *G(c) values for the hydrolysis of* ***MPDA*** *in the presence of ester and amide cosolutes versus the number of CH_2-units in the cosolute molecule*.

2.5.3 Poly(vinylpyrrolidinone) as a cosolute

Finally, the effects of the polymer poly(vinylpyrrolidinone) (PVP) on the hydrolysis of **MPDA**, **BPhT** and **BT** have been investigated. In this way the kinetic effects of a concentrated amount (cluster) of amide bonds can be studied as a simple model for a protein backbone. PVP has a broad variety of applications[45], which can be attributed to its nontoxic nature and the fact that it is very soluble in both water and a large number of organic solvents. It possesses affinity to interact with hydrophobic as well as hydrophilic groups[45]. The solvent effects of PVP have been investigated for two different molecular weights: MW 8,100 and MW 57,500. The results have been analysed in terms of retardation caused per monomer molality of PVP, in order to make a comparison with NMP, which is equivalent to the PVP monomer in terms of number of CH-groups. Clearly, a *pairwise* Gibbs energy interaction parameter cannot be determined, since there are many kinetic probe molecules interacting with one polymer molecule.



PVP

In Figure 2.7 the solvent effects of the high molecular weight polymer have been plotted as a function of monomer molality for the three kinetic probes. Within the experimental error, the results for the low molecular PVP are similar to those shown in Figure 2.7.

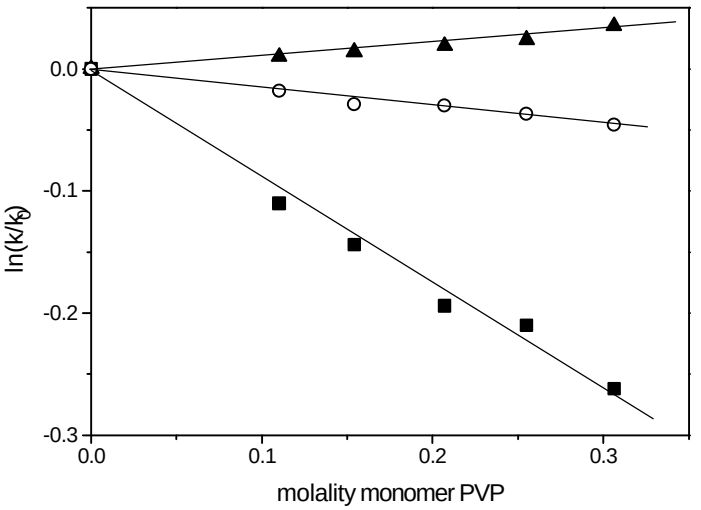


Figure 2.7 *Rate retardations caused by PVP (MW 57,500) relative to the water rate constant (k_0) on the hydrolyses of* ***MPDA*** *(■),* ***BPhT*** *(▲) and* ***BT*** *(O).*

The retardations of the hydrolyses of **MPDA** and **BT** caused by PVP are slightly less than those caused by similar concentrations of NMP. Since the retardations of NMP and PVP are very similar, it can be established that the polymer monomeric units

behave similarly compared with NMP in their interactions with the two probes. In PVP, there is intramolecular hydration shell overlap of the amide units, resulting in slightly smaller kinetic effects. Furthermore, the hydrophobic interactions of the backbone part of the polymer with apolar parts on the probe molecule can be expected to be slightly less than those with NMP, due to crowding and bulkiness of the molecule, which also results in smaller retardations. Remarkably, PVP slightly *increases* the reaction rate of the hydrolysis of (the most hydrophobic probe) **BPhT**. Note that the effect of NMP on the hydrolysis of **BPhT** was also small, although retarding. Thus, for **BPhT** at least, NMP has only little hydrophobic character. As mentioned above, PVP is able to interact with hydrophobic as well as with hydrophilic compounds. Thus, its appearance in aqueous solution may be such, that it consists of hydrophobic and hydrophilic interaction domains and that one probe interacts preferentially with hydrophilic and the other preferentially with hydrophobic domains. Initial state stabilisation by hydrophobic interactions may be sterically impossible, because **BPhT** and PVP are both bulky molecules. They cannot counteract the transition state stabilisation which arises from favourable interactions of the polarised water molecules and the polar amide functionalities of PVP. This lack of initial state stabilisation would then account for the observed rate enhancements. For **BT** and **MPDA** the reaction sites might be somewhat more accessible and initial state stabilisation more pronounced, leading to slight decelerations. Overall, it seems that conformational problems dominate the interactions and specific favourable group interactions play only a minor role in the kinetic medium effects caused by PVP.

In addition, one should bear in mind that it is not realistic to view PVP as a linear arrangement of NMP molecules. A space-filling model of PVP shows that the nitrogen atom of the amide bond is buried between the CH_2-moieties of both backbone and ring system. Hence, polar, stabilising interactions with the activated complex in the transition state take place via a carbonyl rather than via an amide bond. It is difficult to assess whether these are more favourable or not. Since it was previously discussed that amides are better stabilisers of the transition states of the studied reactions, PVP may stabilise the transition state to a lesser extent. Also, it is likely that PVP has lost some of its polar character due to increase in the apparent hydrophobicity of the CH_2-moieties caused by the less extensive amide hydration. This increases the stabilisation of the initial state, leading, however, to kinetic medium effects which are larger than for NMP, which is in contradiction with the observations. Obviously, PVP has not a very disturbing effect on the 3-D-structure of water, the hydrophobic and hydrophilic parts reducing each other's effect on the water structure effectively, and is only just 'noticed' by the kinetic probes.

In conclusion, the kinetic solvent effects caused by PVP are clearly effects which affect the Gibbs energies of both the initial state and the transition state. As long as these interactions have not be quantified separately, it remains difficult to nail down the intermolecular interactions that contribute to and dominate the medium effect of PVP.

2.6. Conclusions

At low molalities, solvent effects of *N*-alkyl-2-pyrrolidinones on the three water-catalysed reactions can be analysed in terms of pairwise interactions. Since more hydrophobic cosolutes retarded the hydrolysis reactions to a greater extent, the kinetic method provides a means of classifying the cosolutes in terms of hydrophobicity. However, hydrophobic interactions do not solely govern or dominate the solvent effects, as was particularly revealed from experiments with different kinetic probes. Noncovalent interactions involving the functional groups (*i.e* amide and ester) are important as well and mainly play a role in the stabilisation of the transition states.

Regarding additivity of functional groups: there is no unique rate retarding effect by a methylene moiety, but its effect depends on the distance from the amide functionality. The intramolecular amide (hydrophilic) and alkyl (hydrophobic) hydration shells of the cosolute molecules overlap. This overlap is destructive since the hydration shells are incompatible. For the first three carbon atoms attached to the amide N-atom there is a reduced contribution of CH_2-groups to the solvent effect, due to extensive amide hydration, which is additive. The hydrophobic effect becomes increasingly more pronounced for hydrophobic moieties which are more than three consecutive carbon atoms remote from a hydrophilic moiety.

A study involving compounds which are structurally related to the *N*-alkyl-2-pyrrolidinones showed how subtle the balance of noncovalent interactions can be.

2.7. Experimental procedures

Materials. p-Methoxyphenyl dichloroacetate (**MPDA**), 1-benzoyl-1,2,4-triazole (**BT**) and 1-benzoyl-3-phenyl-1,2,4-triazole (**BPhT**) were available. Their syntheses had

been carried out according to literature procedures[20,46]. Cosolutes were commercially available (DMA, MAc from Janssen, NiPP, 2PIP from Aldrich, NEP, ValLac from Fluka, NMP, NCHP, BuLac, PVP from GAF, NnBP from Tokyo Kassei Japan). All cosolutes were purified by distillation in vacuo prior to immediate use, except for PVP.

Kinetic measurements. Aqueous solutions for the kinetic measurements were prepared by weight immediately before use. Water was distilled twice in an all-quartz distillation unit. The pH of the solution was carefully adjusted with an aqueous HCl solution with an Orion pH-meter. Between 5-8 µl of a stock solution containing **1** (ca. 10^{-4} M) in acetonitrile (P.A. quality) were injected into 2.5 ml of reaction medium in a quartz cuvette and placed in a thermostated cell compartment (25.0 ± 0.05°C) of a Perkin Elmer λ5 or λ2 spectrophotometer. Pseudo-first-order rate constants were determined by following the change in absorbance at 288 nm (**MPDA**), 273 nm (**BPhT**) or 250 nm (**BT**) using a Perkin Elmer λ2 or λ5 UV/VIS-spectrophotometer. Rate constants for the water reaction were 3.0 x 10^{-3} s^{-1}, 1.24 x 10^{-3} s^{-1}, and 2.07 x 10^{-3} s^{-1}, respectively, in good agreement with literature values[15,16]. The half-lives of these hydrolysis reactions are such, that any possible hydrolysis of cosolutes can be safely neglected.

In general the absorbance did not exceed 0.7. The reaction was followed for about ten half-lives and satisfactory first-order kinetics were observed. Data were converted to rate constants using a commercially available data station. Rate constants were obtained in triplicate and generally reproducible to within 1%. Rate constants for PVP were reproducible to within 3%. Rate constants were determined for at least five different molalities. G(c) values were obtained by a linear regression program. Errors in G(c) values are standard deviations.

2.8. Acknowledgement

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CHAPTER 3

Effects of Cationic Group Hydration on Noncovalent Interactions

3.1 Introduction. Properties of alkylated ammonium bromides in aqueous solution

In the previous chapter the effects of cosolutes containing a polar group were investigated to some extent. Attention is now turned to the effects of cosolutes containing charged groups. The interesting results arising from the study of Noordman *et al*.[1], which revealed for the first time how an anionic group affects the hydrophobic interactions of *n*-alkylsulfates with 1-benzoyl-1,2,4-triazole, initiated a similar investigation of the effects of cationic solutes on intermolecular hydrophobic interactions.

Symmetric tetraalkylammonium halides are an obvious choice since they were previously identified as good models for studying hydrophobic phenomena, in part because of their relatively simple structure, the availability of series of cations with different alkyl chain lengths and their high solubilities in water. Indeed, the solution properties of these 'hydrophobic salts' have been the subject of intense research for more than three decades[2]. The early investigations pointed indirectly to increased ordering of hydration water around the hydrocarbon groups of these ions in water[3]. Later, other experimental studies confirmed this increase in structure of water induced by these hydrophobic salts[4] and up to several years ago[5,6] "structure-making" ("breaking") terminology has been used to explain their aqueous solution properties. More recently, however, experimental results have been interpreted without resorting these concepts[7,8].

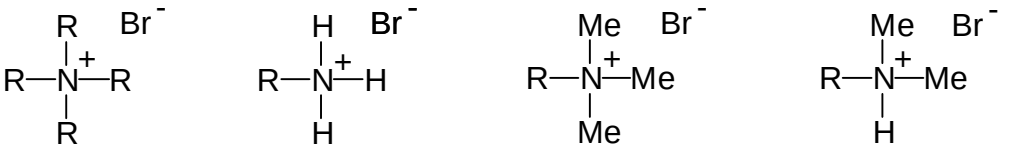
With the goal of obtaining direct evidence for enhanced water-water interactions around these salts, Turner, Soper and Finney[9] systematically studied aqueous solutions of tetramethyl-, tetrapropyl- and tetrabutylammonium halides in aqueous solution by sophisticated neutron diffraction techniques using isotope substitution (NDIS), focusing on the pair correlations between water-water, water-cation and cation-cation. Their results were a breakthrough and indicated clearly that there is little difference in the average 3-D hydrogen-bond structure around the tetraalkylammonium cations, which implies that the structural enhancement of water commonly associated with hydrophobic hydration is small or even absent. This conclusion was also supported by a recent molecular dynamics simulations study of

tetraalkylammonium halides in aqueous solution[10]. In addition, this study suggested that the hydrogen bonds between water molecules in the hydration shell are somewhat shorter and more linear in character than those in the bulk. This is not surprising, since alkylated ammonium salts lead to considerable electrostriction (contraction) of the solvent[11,12,13].

However, water in the first hydration shell of tetraalkylammonium halides is dynamically different from bulk water. The translational motion of water molecules is hindered in the hydration shell, as was shown by water self-diffusion coefficients[14] and NMR studies[15,16,17] and is also in accord with the early discovery of the high viscosities of tetraalkylammonium salt solutions[18].

Ion pairing could have some influence on the hydration structure around the cations, especially since the interactions involving ions in aqueous solution are long-range. However, it is assumed that ion pairing in dilute aqueous solutions containing tetraalkylammonium salts is negligible and that the cations and anions are at least solvent separated and possess independent hydration shells[19].

The aim of the present kinetic study is to investigate the effects of the cationic ammonium hydration on hydrophobic interactions of the solute apolar alkyl groups with the hydrolytic probe 1-benzoyl-1,2,4-triazole (BT) (see Chapter 2) in dilute aqueous solutions. In addition to the tetra-*n*-alkylammonium bromides (1), *n*-alkylammonium (2), *n*-alkyltrimethylammonium (3) and *n*-alkyldimethylammonium (4) bromides have also been used as cosolutes.



1 2 3 4

The questions prompting this study were:

1. Are these ionic solutes really hydrophobic ions, *i.e* are they able to interact via hydrophobic interactions with BT?
2. What are the effects of varying the alkyl group R from methyl to *n*-hexyl on hydrophobic interactions with the kinetic probe in terms of additivity of group interactions?
3. In connection with question 2, how extensive is the ammonium group hydration and what is its effect on hydrophobic interactions?

3.2 Kinetic solvent effects of alkylated ammonium bromides on the water-catalysed hydrolysis of 1-benzoyl-1,2,4-triazole

The kinetic medium effects of the hydrophobic salts 1-4 on the hydrolysis of 1-benzoyl-1,2,4-triazole at pH 4 and 25°C have been measured at different molalities. Generally, rate constants were obtained up to a concentration of 1 molal of added cosolute, except for *n*-hexylammonium and *n*-hexyldimethylammonium bromide, which were measured up to a limit of 0.5 molal, because these salts showed evidence for aggregation above this concentration. These concentrations are expected to allow the formation of at least one independent layer of water around each cation[20,9e]. Like anionic cosolutes[1], the cationic alkylammonium bromides retard the hydrolysis of BT. As an example, the medium effects on the neutral hydrolysis of BT, expressed as the dependence of $\ln(k/k_0)$ on the molality of added tetra-*n*-alkylammonium bromides, are shown in Figure 3.1. The tetra-*n*-alkylammonium bromides produced excellent linear correlations between $\ln(k/k_0)$ and the molality of added salt indicating pairwise (*i.e*. 1:1) interactions with BT. The salts 2-4 showed a similar linear dependence on molality. G(c) values were obtained from the slopes of these plots and are compiled in Table 3.1.

In terms of the SWAG approach[21], each functional group in one molecule interacts with every functional group in the other molecule.

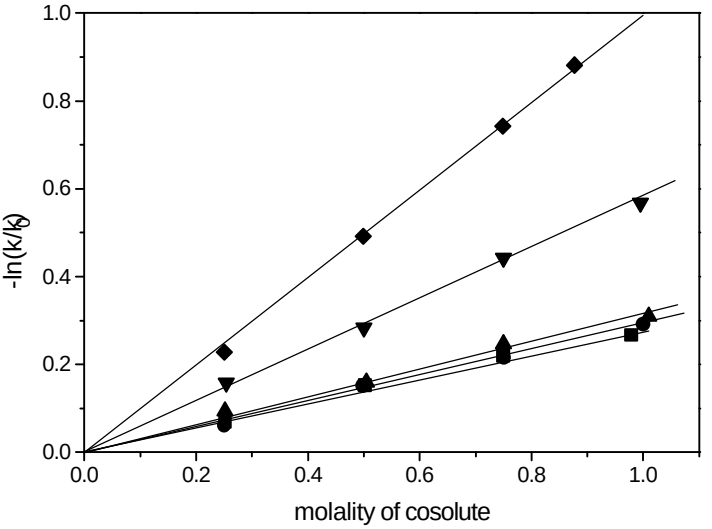


Figure 3.1 *Kinetic medium effects on the pseudo-first-order rate constant for hydrolysis of BT at pH 4 and 25 °C, plotted as $\ln(k/k_0)$ versus the molality of ammonium bromide ( ), tetramethylammonium bromide (▲), tetraethylammonium bromide (●), tetra-n-propylammonium bromide (▼) and tetra-n-butylammonium bromide (◆).*

Each of these interactions has a characteristic effect on the pairwise Gibbs energy interaction parameter G(c). It has been shown that the SWAG-approach is also applicable to the interactions between electrolytes and non-electrolytes[22], amongst

others between tetraalkylammonium bromides and DMF. In the case of alkyl-substituted ammonium bromides, G(c) is composed of contributions due to *n*

Table 3.1. *G(c) values (J kg mol^{-2}) for tetra-n-alkylammonium, n-alkylammonium, n-alkyldimethylammonium and n-alkyltrimethylammonium bromides as cosolutes in the water-catalysed hydrolysis of 1-benzoyl-1,2,4-triazole. Standard deviations in G(c) in parenthesis.*

R	$R_4N^+Br^-$	$RNH_3^+Br^-$	$RN^+H(Me)_2Br^-$	$RN^+(Me)_3Br^-$
H	-307 (7)	-307 (7)	n.d[a].	n.d.
methyl	-324 (4)	n.d.	n.d.	-324 (4)
ethyl	-389 (15)	n.d.	n.d.	n.d.
n-propyl	-655 (7)	-471 (26)	-415 (8)	-436 (6)
n-butyl	-1190 (13)	-486 (4)	-527 (16)	-512 (14)
n-pentyl	n.d.	-631 (6)	-625 (10)	-575 (13)
n-hexyl	n.d.	-735 (7)	-696 (8)	-782 (6)

[a]Not determined

methylene moieties in the alkyl chain, the ammonium ion and the bromide ion. In the case of perfect additivity, the increment in G(c) of the cosolutes depends solely on the difference in the number of CH_2-groups. In Figure3.2, the G(c) values for the tetra-*n*-alkylammonium bromides are plotted versus the number of CH_2-groups (where the contribution of a CH_3-group equals the contribution of 1.5 CH_2-group and the contribution of 3 CH-groups[23]). Obviously, there is no constant contribution of the CH_2-group to the kinetic medium effect throughout the series. Hardly any effect on the G(c) value is observed when the alkyl chain length increases from zero to two carbon atoms per chain; *i.e.* from ammonium bromide to tetraethylammonium bromide. Most probably, these methylene groups are not available for hydrophobic interactions with the hydrophobic groups of BT. This sensitivity is due to the hydrophilic hydration sphere of the ammonium headgroup, which extends to the third carbon atom in the *n*-alkyl chain. The influence of ionic hydration shells on the

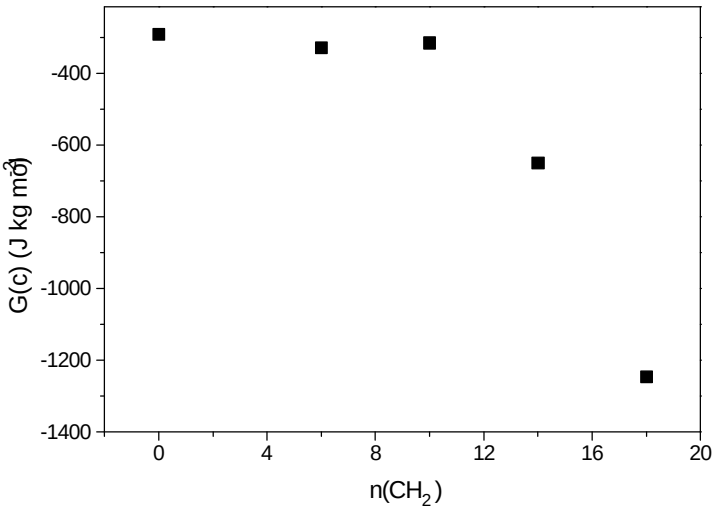


Figure 3.2 *Medium effects of tetra-n-alkylammonium*

bromides on the hydrolysis of BT at 25 °C and pH 4. Plot of G(c) values versus number of CH_2-moieties in the cosolute.

hydrophobicity of nearby methylene groups agrees very well with kinetic solvent effect studies of aqueous solutions containing sodium *n*-alkylsulfates[1], *n*-alkylpyrrolidinones [24] and α-amino acids[25]. Gianni *et al.*[26] recently analysed partial molal volumes of organic electrolytes at infinite dilution by a simple additivity scheme from which they also concluded that the region of influence of a polar group does not extend beyond the β carbon atom of the alkyl substituent. The kinetic observations are also supported by Hirata *et al*.[27], who investigated the micellisation behaviour of double-headed surfactants in which two quaternary ammonium species ($C_{10}H_{21}N^+(CH_3)_2$) are linked by a hydrocarbon spacer. The results indicated that surfactants having spacers of 4 methylene moieties and shorter behave as normal micelle forming surfactants whereas 6 methylene moieties and longer show deviating behaviour. Methylene moieties in the short spacers do not cause these deviations because they are not significantly hydrophobically hydrated due to the strong hydrophilic hydration of the two ammonium head groups.

Despite the absence of hydrophobic interactions between the short-chain alkylammonium bromides and BT, a retardation of the hydrolysis is observed. For the longer-chained compounds the retardations are mainly the result of a dominant stabilisation of the more hydrophobic initial state of the hydrolysis by these cosolutes through hydrophobic interactions (these alkylated ammonium salts are generally viewed as hydrophobic species[28,29]). The retardations caused by the short-chain compounds must have another origin (even though there is convincing evidence that, for example, the tetramethylammonium cation is a hydrophobic species according to neutron diffraction experiments[30]). Even ammonium bromide has a rate retarding effect on the neutral hydrolysis of BT, G(c) = -307 (7) J kg mol^{-2}, despite the absence of any hydrophobic groups. One would actually anticipate an increase in rate due to a stabilisation of the polar transition state by favourable electrostatic interactions with the charged group, which is not screened by alkyl groups in the case of ammonium bromide. It is noted, however, that it was observed previously that an anionic group can have a retarding effect on the hydrolysis of BT. In the case of sodium *n*-alkylsulphates[1], G(OSO_3^-) is approximately -600 J kg mol^{-2}. Recently, Berendsen *et al.*[31] calculated the proton transfer rate constant for the rate determining step in the water-catalysed hydrolysis of a carboxylic ester, *p*-methoxyphenyl dichloroacetate, by means of MD simulations and Density Matrix Evolution (DME). The water-catalysed hydrolysis of this substrate proceeds via the same mechanism[32] as the water-catalysed hydrolysis of BT. They observed that only a few of the many possible water orientations which lead to reaction can account for the experimental rate constant. As a possible explanation for the observed

retardations in the presence of the (alkylated) ammonium bromide(s) it is suggested that the strict orientational requirements for the water molecules in the activated complex are more pronounced when these water-demanding salts are added to the aqueous solution. Ammonium bromide is extensively hydrated, mainly through hydrogen bonding to the four polarised N-H groups, and competes with the transition state for water molecules, *i.e*. a salting out effect is operative particularly for the transition state. However, in view of the MD simulation[31], a more likely explanation lies in the reduced (translational) mobility of the water molecules due to the presence of the cosolutes, *i.e*. the water molecules in the hydration shells are less dynamic and it simply takes more time to find the configuration necessary for the reaction to occur. The larger negative contribution to the G(c) value by the OSO_3^--group[1] might be rationalised in terms of the reduced shielding of the charge relative to that for the alkylated ammonium ions. Consequently, the anions have a larger influence on the 3D hydrogen-bond network of water than the cationic ammonium bromides. However, this influence does not reach further than the influence of the cations; both affect the hydration of the alkyl groups up to the γ-C atom.

For the other three series of ammonium bromides G(c) values have been obtained for compounds with alkyl chains longer than two carbon atoms only (see table 3.1) and therefore the results cannot provide additional evidence for the assumption that the cationic charge reduces the apparent hydrophobicity of neighbouring methylene moieties. However, outside the destructive influence of the cationic charge, additivity of CH_2-group interactions is more likely to occur. In Figure 3.3, all G(c) values have been plotted versus the total number of CH_2-groups. The relationships are fairly linear (except for the tetraalkylammonium bromides), indeed suggesting additivity of CH_2-group interactions with BT for methylene moieties which are independently hydrated. The slopes of these plots give an indication for the strength of these noncovalent pairwise group interactions and are compiled in Table 3.2. As can

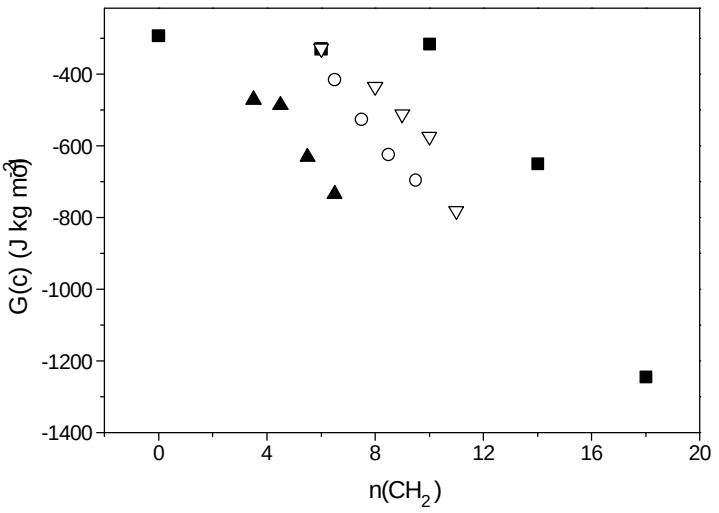


Figure 3.3. *G(c) values for the tetraalkylammonium (■), alkylammonium (▲), alkyldimethylammonium (O) and alkyl*

trimethylammonium bromides (∇) as a function of the total number of CH_2 groups in the solutes.

be seen, the slopes ($G(CH_2)$) are the same within experimental error, except for

Table 3.2 *Pairwise Gibbs energy interaction parameters for the CH_2-group in the various series of cosolutes.*

Solutes	$G(CH_2)$[a] J kg mol^{-2}
$R_4N^+ Br^-$	116 (19)
$RNH^+_3 Br^-$	125 (12)
$RN^+H(Me)_2 Br^-$	94 (7)
$RN^+Me_3 Br^-$	110 (24)
alcohols	90 (3)[b]

[a]Only data for solutes with alkyl substituents larger than ethyl have been taken into account. [b]Taken from ref. 33.

the alkyldimethyl ammonium bromides. The $G(CH_2)$ values are slightly higher than the value obtained for pairwise interactions between linear alcohols and BT, but this is not surprising since that value is based on the difference between ethanol and 1-propanol and it is now clear that the alkyl moieties of those two solutes are not completely independently hydrated, due to the influence of the hydroxyl group.

To illustrate more clearly that only methylene moieties outside the hydration sphere of the ionic group (*i.e*. further than two carbon atoms away from the ionic group) are available for hydrophobic interactions with the substrate, the G(c) values for *n*-alkyldimethylammonium bromides are plotted again versus the number of CH_2-moieties in Figure 3.4, but now leaving space for extrapolation towards zero methylene groups. If the argumentation above is correct, the group interaction

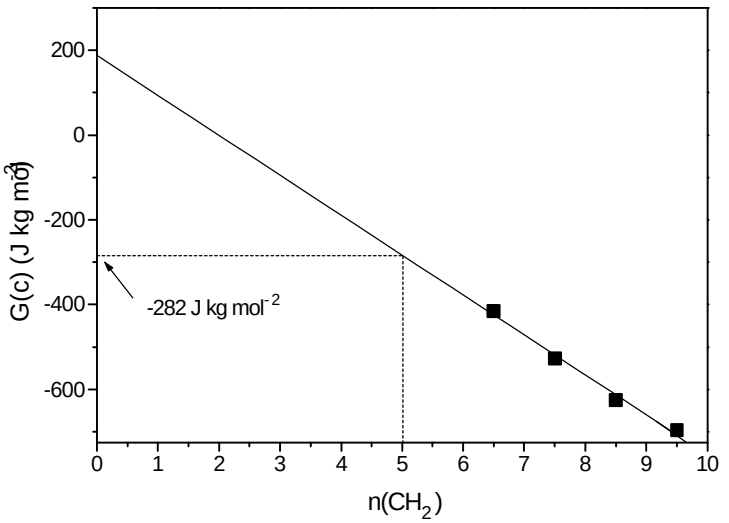


Figure 3.4 *Medium effects of n-alkyldimethylammonium bromides on the hydrolysis of BT at 25°C. Plot of G(c) versus number of CH_2 moieties. Extrapolation according to the SWAG-approach (5 CH_2-moieties = $CH_2CH_2N(H)Me_2$).*

parameter for the $CH_2CH_2N^+(H)Me_2Br^-$-group should be similar to the G(c) value for ammonium bromide. This value is obtained by extrapolation of the linear correlation shown in Figure 3.4 to where n(CH_2) equals five. This gives a value for G($CH_2CH_2N^+(H)Me_2Br^-$) of -282 J kg mol^{-2}. This is in satisfactory agreement with the experimental G(c) value for ammonium bromide of -307 (7) J kg mol^{-2}, again supporting that hydrophobic groups in close proximity to a polar group are masked for hydrophobic interactions with the kinetic probe. A similar extrapolation of the data for the *n*-alkylammonium and *n*-alkyltrimethylammonium bromides gives values for G($CH_2CH_2N^+H_3$) of -300 J kg mol^{-2} and for G($CH_2CH_2NMe_3$) of -245 J kg mol^{-2}, respectively. The deviation of the latter value for *n*-alkyltrimethylammonium bromides is probably due to the less pronounced apolar group additivity (*i.e.*, a larger error in extrapolation).

Now that it is established that the noncovalent interactions of alkylated ammonium bromides with linear alkyl chains shorter than 3 linear C-atoms with BT are governed primarily by the headgroup hydration, it is worthwhile to plot the alkyl chain length of the salts 1-4 versus the G(c) value, irrespective of the substitution pattern of the ammonium head group (*i.e*. how many methyl groups (or even ethyl groups, in the case of tetra-*n*-alkyl ammonium bromides) it contains in addition). This is shown in Figure 3.5. As anticipated, the data for the various series of cosolutes nearly overlap each other. Thus, once more, it is illustrated that the kinetic probe hardly distinguishes between an ammonium, a methyl-, dimethyl- or trimethylammonium head group and even a triethylammonium group in the case of tetraethylammonium bromide. In the case of tetra-*n*-propylammonium bromide, the cationic hydration looses its dominance. Clearly there is a difference between solutes bearing one or four *n*-propyl groups and this distinguishes the tetra-*n*-alkylammonium salts from the other series.

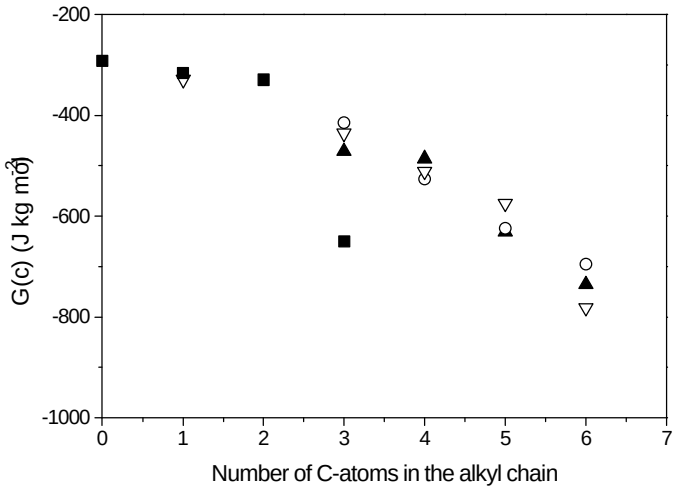


Figure 3.5. *G(c) parameters for tetraalkylammonium (■),*

In view of these results it is expected that the alkyl*ethyl*-ammonium bromides will show overlap with the data in Figure 3.5. Unfortunately, such data are not available.

As mentioned in the introduction, there is a vast amount of thermodynamic data

alkylammonium (▲), alkyldimethylammonium (O) and alkyl trimethylammonium bromides (∇) as a function of the number of C-atoms in the linear alkyl chain which is varied in length.

(partial molar properties, enthalpic self-interaction coefficients, hydration numbers) available for aqueous solutions containing, in particular, tetraalkylammonium bromides[2]. It is of interest to correlate these solution data with the kinetic data presented in this chapter, to investigate whether the pairwise interactions are specific for the hydration of alkylated ammonium compounds or whether they rather reflect specific interactions with BT.

However, partial molar volumes[26,34], heat capacities[35], compressibilities[34,36] and expansibilities[35] do not show total independence of the R substituent up to carbon atom three for the tetraalkylammonium salts. It may be that the *intrinsic* contributions to the partial molar properties are considerable in the case of these salts, *i.e*. that the hydration contribution does not dominate the partial molar property. The kinetics however, purely reflect the *hydration characteristics* of the salts.

A final remark should be made considering the effect of the counterion. Kinetic experiments with alkylated ammonium chlorides have been performed and did not give different results from those for the bromides. Therefore, it is concluded that there is no cation-anion pairing, because the anions are differently hydrated and an effect on the kinetics would be expected.

Moreover, the hydrated anions do not interact with BT, for similar reasons. Thus, the observed rate effects are caused by the cations only.

3.3 Conclusion

Analysis of G(c) parameters suggests that the ionic hydration shell of the ammonium headgroup in *n*-alkylated ammonium bromides is incompatible with the hydration shell of the alkyl groups attached to it. The water molecules in the ionic hydration shell will be oriented differently and have stronger (electrostatic) interactions with the solute than the water molecules in the hydrophobic hydration shell. The ionic hydration shell reduces the apparent hydrophobicity of methylene groups within a distance of 3 consecutive carbon atoms. Hydrophobic interactions are only observed for *n*-alkylated ammonium compounds with alkyl groups with more than two carbon atoms. For two or fewer carbon atoms, negative G(c) values are presumably caused by a reduction of the water (translational) mobility during the activation process, which requires the specific and precise orientation of two water molecules.

For those solutes containing methylene groups outside the ionic hydration sphere, additivity of methylene group interaction is observed.

3.4 Experimental procedures

Materials. 1-Benzoyl-1,2,4-triazole was available. Its synthesis has been described in the literature[32a]. The *n*-alkylamines, tetra-*n*-alkylammonium bromides and methyl bromide were purchased from Janssen Chimica, Fluka and Sigma. The *n*-alkylamines were distilled before use.

The *n*-alkylammonium bromides and the dimethyl-*n*-alkylammonium bromides were prepared in situ by acidifying the corresponding *n*-alkylamines and dimethyl-*n*-alkylamines, respectively, with aqueous hydrogen bromide to pH 4. The *n*-alkyldimethylamines were synthesised by Esch-Weiler Clark methylation[37] of the corresponding *n*-alkylamine.

The *n*-alkyltrimethylammonium bromides were synthesised by methylation of the corresponding *n*-alkyldimethylamine with methyl bromide -20 °C in acetone. The acetone was carefully removed via a steel wire under moderate nitrogen pressure. The salts were dried in a drying pistol until no further loss in weight was observed. The purity of the tetra-*n*-alkyl- and the *n*-alkyl-trimethylammonium bromides was checked by ^{1}H-NMR and ^{13}C-NMR spectroscopy.

Kinetic measurements. The kinetic experiments have been carried out according to the procedures described in Section 2.7. Rate constants were reproducible within 2%. Reaction rate constants at each molality were measured in triplicate. Kinetic data for each set of solutions were determined at least 4 different molalities.

3.5 Acknowledgement

Peter Hol synthesised the alkylammonium bromides and performed the kinetic measurements reported in this chapter within the framework of the research project of his masters study. He is also acknowledged for the pleasant cooperation during this period.

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CHAPTER 4

Effects of Zwitterionic α-Amino Acid Hydration on Noncovalent Interactions

4.1 Introduction

Now that general ideas about the influence of anionic[1] and cationic[2] solute hydration on hydrophobic interactions involving (substituted) 1-acyl-1,2,4-triazoles have been developed, it is of interest to examine the combined effects of two opposite charges in the same molecule, like those in zwitterionic α-amino acids. The kinetic medium effects of alkylammonium bromides on the hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole, which were presented in the previous chapter, provide a starting point for the investigation of the kinetic medium effects of zwitterionic α-amino acids. In this context it would have been desirable to have kinetic data for a series of carboxylic acids as well, but these solutes catalyse the amide hydrolysis as general bases.

As was pointed out in Chapter 1, it is of utmost importance to obtain quantitative data about α-amino acid hydration in order to be able to qualify and quantify the noncovalent interactions that play a role in biologically relevant processes such as protein folding. In view of the fact that hydrophobic interactions between α-amino acid side chains contribute to the driving forces for protein folding[34], the choice of the hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole (BPhT) as a kinetic model reaction through which these noncovalent interactions can be measured is justified, since in the past solvent effects on this reaction have been explained in terms of hydrophobic interactions (see Section 2.3). Furthermore, the kinetic probe contains an amide functionality, which is a model for the protein peptide bond.

4.2 Kinetic solvent effects on the neutral hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole in dilute aqueous α-amino acid solutions

In this chapter the kinetic medium effects of a series of α-amino acids and derivatives on the neutral hydrolysis of BPhT at pH 4 and 25°C are reported. The mechanism of this hydrolysis reaction has been discussed in Chapter 2. Needless to say that the pH of the aqueous solutions must be kept as constant as possible in view of the acid/base properties of the carboxylic and amino groups. At pH 4 the concentration of zwitterions

is about 50 times higher than that of cations, so the observed medium effects are primarily determined by the zwitterions (for about 98%). The fluctuation of this concentration ratio among the different solutes is small because the pK_A's of the investigated α-amino acids differ only slightly[5]. The choice of α-amino acids which were examined was limited by their solubility restraints.

The kinetic medium effects are converted into pairwise Gibbs energy interaction parameters using the thermodynamic approach for the evaluation of medium effects on rate constants (Section 2.2).

The resulting G(c) values are critically discussed in terms of functional group interactions and intramolecular hydration shell overlap effects. Results for some α-amino acid derivatives have shed more light on the contributions of the different functional groups to the kinetic medium effect.

Additional information about the types of noncovalent interactions which play a role in the recognition processes was obtained by determination of isobaric activation parameters for the hydrolysis of BPhT in some dilute α-amino acid solutions.

4.2.1 Analysis of the kinetic solvent effects

The effects of α-amino acids on the kinetics of BPhT have been measured up to 1 molal for sufficiently soluble α-amino acids and up to maximum solubility for the less soluble ones. As demanded by the theory for analysis of pairwise interactions as described in Section 2.2, the natural logarithm of the rate constant varied linearly with the molality of the cosolute. At higher concentrations (m > 1.5) deviation from linearity was observed, most likely due to triplet and higher order interactions. In Figure 4.1 the effects of glycine, proline, α-alanine, α-valine and α-serine are visualised. G(c) values were obtained from the slopes of these plots using the equation in Section 2.2. In this equation the practical osmotic coefficient of water can still be approximated by 1 in the

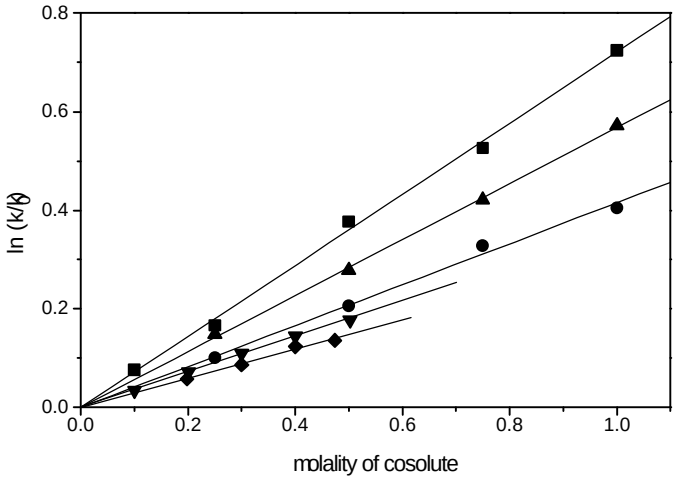


Figure 4.1 *Kinetic medium effects of glycine (■), proline (▲), alanine (●), valine (▼) and serine (◆) on the hydrolysis of BPhT.*

Table 4.1. *G(c) values[a] (J kg mol^{-2}) for α-amino acids at 298.15 K and pH 4.*

α-Amino acid	α-C-substituent	pK_a (α-CO_2H)	G(c)
glycine	hydrogen	2.35	875 (21)
alanine	methyl	2.35	558 (16)
α-amino-*iso*-butyric acid	2x methyl	2.36	429 (17)
proline	-$(CH_2)_3$-	2.00	632 (15)
α-amino-*n*-butyric acid	ethyl	2.55	565 (15)
norvaline	*n*-propyl	2.30	556 (15)
valine	*iso*-propyl	2.29	467 (9)
leucine	*iso*-butyl	2.33	518 (21)
isoleucine	*sec*-butyl	2.32	499 (20)
lysine	$(CH_2)_4NH_3^+$	2.16	642 (29)
threonine	$CH(CH_3)OH$	2.09	445 (9)
serine	CH_2OH	2.19	383 (6)
asparagine	CH_2CONH_2	2.02	436 (8)
3-phenylserine	CHC_6H_5OH	n.a[b].	-479 (25)
phenylalanine	$CH_2C_6H_5$	2.20	-709 (6)

[a]*Errors in parenthesis.* [b]*Not available.*

presence of α-amino acids[6]. The G(c) values of a series of α-amino acids are displayed in Table 4.1. The most striking feature reflected by these data is the acceleration of the hydrolysis reaction in the majority of the aqueous α-amino acid solutions compared to the reaction in pure water. Interestingly, this is the first time that substantial rate enhancements for this hydrolysis reaction in the presence of small amounts of cosolutes are observed. An obvious explanation would imply general-base catalysis by the carboxylate group. This could be checked by measuring the rate constants in α-amino acid solutions with constant concentration over a large pH range. However, the neutral hydrolysis is restricted to pH values between pH 3.5 and 5, where the differences in reaction rates due to changes in ionisation of the carboxyl group are outside the kinetic detection limit. If, however, general-base catalysis would interfere with the medium effect, a linear dependence of the logarithm of the second-order rate constant on the pK_A of the α-amino acid is expected. Such a Brönsted relationship is not found[a]. To find out whether any change in reaction mechanism takes place in dilute aqueous α-amino acid solutions, solvent deuterium kinetic isotope effects have been measured for pure water and for an aqueous solution containing Gly. The values indicate that the

mechanism of hydrolysis is unchanged upon replacing water for an aqueous solution containing Gly:

solvent	$k(H_2O)/k(D_2O)$
water, pH 4	2.69 (± 0.01)
0.5 molal Gly (aq), pH 4	2.49 (± 0.05)

Finally, in case of general-base catalysis, plots of k_{obs} versus the molality of cosolute should yield a linear relationship with a slope equal to k_{cat}. Such a relationship was not found either. This indicates that the observed kinetic effects are not governed by general-base catalysis of the amino acid carboxylate group, but involve medium effects instead.

In view of the zwitterionic character of the α-amino acids it seems likely that an electrostatic interaction between the dipolar activated complex in the transition state and the zwitterions is operative, presumably via polarised water molecules. Electrostatic stabilisation of the transition state is also likely in view of the increased relative permittivities of aqueous α-amino acid solutions[7]. Typically, a 1 molar aqueous Gly solution has a relative permittivity of 102 relative to 78.4 for water. Previously, kinetic medium effects, including kinetic salt effects[8c], on the water-catalysed hydrolysis of *p*-nitrophenylsulfonylmethyl perchlorates have been interpreted in terms of polarisation of water molecules as well[8]. In Section 4.4, the water polarisation around the charged solutes will be discussed in more detail.

[a]A Brönsted value of 0.35, together with the rate constant of BPhT in pure water, can be used to estimate catalytic rate constants (log $k_B = -\beta\ pK_{BH} + c$). This Brönsted value is reasonable when compared to the hydrolysis of other, structurally related 1-acyl-3-substituted-1,2,4-triazoles, which show β values between 0.34 and 0.36 for sodium alkanecarboxylates[9]. Using the rate constant of BPhT in pure water, the rate equations thus obtained yield theoretical values for k_{obs} ($k_{obs} = k_0 + k_B[B]$), which are clearly different from the experimental values. For example, the experimental versus calculated pseudo-first order rate constants for the hydrolysis of BPhT in aqueous solutions containing 1 molal of α-amino acid are (times 10^3 in s^{-1}): 2.56 v. 1.88 (Gly), 2.06 v. 1.73 (Pro), 1.92 v. 1.85 (Val), 1.65 (extrapolated) v. 1.81 (Ser).

4.2.2 Hydrophilic α-amino acids

Despite the lack of a straightforward correlation, it is possible to identify, at least to a certain extent, the structural features of the α-amino acids which determine the G(c) values. As is shown in Table 4.1, glycine accelerates the rate of the hydrolysis enormously. Most likely, the central methylene group is not available for participating in hydrophobic interactions with the initial state of the reaction. Due to the presence of both

extensive ionic hydration shells the hydrophobic hydration shell of the methylene group is badly developed. A Monte Carlo computer simulation of the glycine zwitterion[10] showed that the coordination number of the central methylene group in glycine is far less than predicted[11]. It has also been observed that Gly is found primarily in the solvent-exposed regions in proteins[12] (*i.e* is not buried in the apolar interior), which is consistent with its hydrophilicity.

The ionic hydration shells in zwitterionic α-amino acids do not only interfere with the apolar hydration shell of the side chain, but also with each other[13,14,15], up to 5[16] or even to 6[17] intervening CH_2-moieties. Actually, the pressure and temperature dependences of the apparent molar characteristics of glycine qualitatively resemble those of sodium chloride[18], a simple electrolyte. In view of the above observations it is reasonable to assume that the hydration shell of glycine is controlled predominantly by electrostatic interactions between water molecules and the charged termini. Clearly, the kinetic medium effect for Gly is in agreement with these observations and mainly determined by favourable interactions with the activated complex.

Proline also induces a large rate acceleration. Comparison of Pro with other common α-amino acids is not fully justified, because of its cyclic structure and reduced hydrogen bonding capabilities at the amino terminus. The high affinity for water of this imino acid is likewise reflected by its position in protein structures; proline does not fit into α-helices and ß-turns and often shows up at the ends of these secondary structures[19]. Consequently, Pro is often found in the solvent-exposed regions. *Gibbs et al.*[20] ascribed the hydrophilicity of Pro to the fact that the transfer of a Pro residue from the vapour to the aqueous phase is entropically more favourable than that for norvaline, the linear analogue of proline. This observation is explained by the relatively small loss of internal mobility of the ring system when entering the aqueous medium as compared to the flexible linear chain. The loss of enthalpy due to the reduced hydrogen bonding ability at the amino terminus is probably more than compensated for by this effect. Another possible explanation for the hydrophilicity of proline could be the reduced accessibility of the inside region of the apolar ring for hydrophobic interactions. A similar effect has been observed for cyclopentanol. For this cyclic alcohol, the G(c) value is lower compared to that for linear alcohols with the same number of CH_2-groups[21].

4.2.3 Apolar side chains

Attention is now turned to a comparison of α-amino acids with non-branched alkyl chains; α-alanine, α-amino-*n*-butyric acid and norvaline, with methyl, ethyl, and *n*-propyl side chains, respectively. The G(c) values are 558, 565 and 556 J kg mol^{-2}, respectively,

and are equal within experimental error. The reduction in rate constant relative to glycine was anticipated in view of favourable side chain interactions with the initial state which are of hydrophobic nature. However, the reactivity of BPhT is not sensitive to the polarity of the α-substituent. The pattern shows that hydrophobic interactions of the solute (at least up to three aligned carbon atoms) with BPhT play no significant role. This would lead to the notion that they are overwhelmed by interactions mediated by the ionic groups. Even side chains up to four carbon atoms, (though branched; Leu and Ile), show only a minor effect when compared to the shorter chains. These results are consistent with the kinetic results for the alkylammonium bromides[2] (Chapter 2) and for sodium *n*-alkylsulfates[1] as cosolutes. The complete camouflaging of hydrophobic interactions is not surprising because the α-amino acids have two ionic hydration shells which influence the interactions of the apolar part of the molecule to a long range.

The dominance of the ionic hydration over the hydrophobic hydration in α-amino acids is well-documented in the literature and has been investigated by different methods[22]. It is believed that the dominance of electrostatic solvent-solute interactions is mainly operative in the first hydration layer[23].

In short, hydrophobic interactions appear to play only a minor role in the effects caused by most aliphatic α-amino acids. Consequently, there is no simple relationship between the G(c) value and side-chain hydrophobicity as obtained from several hydrophobicity scales for α-amino acids[24].

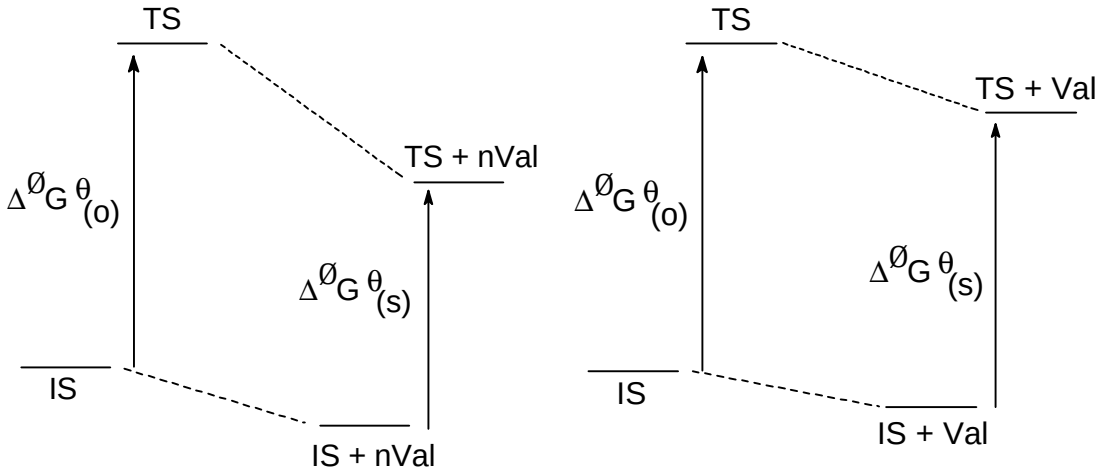
Information about the individual effects of the ionic groups is not revealed by the results obtained from these cosolutes.

4.2.4 Isomeric alkyl side chains

α-Amino-*iso*-butyric acid (Aiba) and α-amino-*n*-butyric acid (Aba) are isomeric cosolutes. A comparison of their rate-enhancing medium effect, G(c) = 429 J kg mol^{-2} and G(c) = 565 J kg mol^{-2}, respectively, is worthwhile. These values indicate that when the α-carbon atom is substituted twice, the effect on the rate of hydrolysis is larger compared to that of the mono-substituted isomer. The most reasonable explanation consists of a combination of two effects. The hydrophobic hydration shells of both methyl groups may develop to a larger extent, due to an increase of the Me-C_α-Me angle by mutual repulsion of the methyl groups. This occurs at the expense of the hydrophilic hydration shells of the carboxylate and ammonium groups, which overlap more extensively due to a reduced N-C_α-C bond angle (*gem*-dimethyl effect[25]). In other words, the relatively small acceleration by Aiba is caused by decreased stabilising interactions with the transition state and increased stabilising hydrophobic interactions with the initial

state. Furthermore, Aiba is conformationally restricted[26]. Since the stabilisation of the transition state via hydration shell overlap may require specific orientations of the α-amino acids, it is possible that Aiba cannot adopt the favourable conformations leading to a stabilisation as significantly as its isomer.

Valine and norvaline, with *i*-propyl and *n*-propyl side chains, respectively, are isomers as well, but norvaline (G(c) = 556 J kg mol^{-2}) is more effective in accelerating the hydrolysis reaction than valine (G(c) = 467 J kg mol^{-2}). Again this difference may be due to intramolecular overlap of hydrophobic and ionic hydration shells which is more pronounced in the case of valine. In view of the kinetic results presented in Chapter 5 for these isomers, it seems likely that the difference in kinetic medium effects by valine and norvaline is dominated by a transition state effect. Norvaline is more hydrophobic than valine[27], and a lower G(c) value would be expected. That the opposite is found must therefore be ascribed to a stabilisation of the transition state through electrostatic interactions with the ionic hydration shells, which is less pronounced for valine than for norvaline (see Scheme 4.1).



Scheme 4.1 *Interactions of norvaline (nVal) (left) and valine (right) with the initial state and activated complex for the hydrolysis of BPhT, where* $D\,G^{q}_{(o)}$ *and* $D\,G^{q}_{(s)}$ *are the Gibbs energies of activation for the hydrolysis in pure water and in aqueous solution containing cosolutes, respectively.*

A similar trend was anticipated for the isomers leucine and isoleucine. Ile has a methyl group on the β-C atom, like Val. Leu and nVal, however, do not have this methyl group. There is a tendency for Ile to accelerate the hydrolysis less than Leu, but since the G(c) values for Ile and Leu are the same within experimental error, there is insufficient proof to nail this down. On the whole, it seems that branching of the side chain near β-C has a more destructive effect on the ionic hydration shell(s) than chain elongation or branching on γ-C.

4.2.5 Side chains with polar character

At first sight the results obtained for serine and threonine appear unexpected because a hydroxyl group can interact more favourably with the activated complex than with the reactant and a positive group contribution to the G(c) is anticipated (as observed in the case of alcohols as cosolutes[28]). In contrast, serine and threonine, with a methanol- and ethanol-like side chain, respectively, are the weakest accelerators among the α-amino acids examined. The polar hydration shell of the hydroxyl group is probably well-developed and at the expense of the ionic hydration shell, or at least it interferes with those shells more destructively than the apolar hydration shell does. Serine shows a lower G(c) value than threonine even though threonine is the more hydrophobic α-amino acid of the two[27]. Presumably, the hydroxyl hydration, and therefore its destructive effect on the ionic hydration, is reduced in the presence of the side chain methyl group.

Asparagine, with a $-CH_2CONH_2$ side chain, has a G(c) value slightly higher than that of serine ($-CH_2OH$), which suggests that the destructive effect of an amide functionality on the carboxylate hydration is less than that of an alcohol moiety.

Lysine has an apparent high G(c) value. General-base catalysis by the amine group may be involved, although this group is largely protonated at pH 4. No further steps were undertaken to investigate this possibility.

4.2.6 α-Amino acid derivatives and related compounds

The above results show that the pairwise Gibbs energy interaction parameters of α-amino acids and BPhT cannot be analysed in terms of the group additivity approach as formulated by Savage and Wood[29]. This failure can be rationalised in terms of a disturbing effect of the hydrated charges present in the cosolute.

To obtain more insight into the individual effects exerted by the ammonium and carboxylate groups several α-amino acid derivatives have been studied (Table 4.2). From measurements where the carboxylate is functionalised to an amide or ester group, it is obvious how important the role of this ionic group is in governing the kinetic medium effect. A decrease in reaction rate compared to the pure water reaction is observed for glycine ethyl ester and glycinamide. Electrostatic interactions between

Table 4.2 *G(c) values[a] (J kg mol^{-2}) for α-amino acid derivatives and related compounds at 298.15 K and pH 4.*

α-Amino acid derivative	**Structural formula**	**pK_A**	**G(c)**
N-methylglycine (sarcosine)	$(Me)H_2N^+CH_2CO_2^-$	2.21	555 (11)
N,N-dimethylglycine	$(Me)_2HN^+CH_2CO_2^-$	2.08	619 (10)
N,N,N-trimethylglycine (betaine)	$(Me)_3N^+CH_2CO_2^-$	1.83	730 (14)
glycine ethylester	$H_3N^+CH_2CO_2Et\ Cl^-$	-	197 (2)
glycinamide	$H_3N^+CH_2CONH_2\ Cl^-$	-	-148 (25)
alaninamide	$H_3N^+CHMeCONH_2\ Cl^-$	-	-234 (6)
phenylalaninamide	$H_3N^+CH(CH_2Ph)CONH_2$	-	-1869 (25)
ammonium cyano acetate	$H_4N^+\ NCCH_2CO_2^-$	2.40	221 (4)
sodium cyano acetate	$Na^+\ NCCH_2CO_2^-$	2.40	97 (3)
amino methane sulphonic acid	$H_3N^+CH_2SO_3^-$	ca. -1	ca. 200

[a]*Errors in parenthesis.*

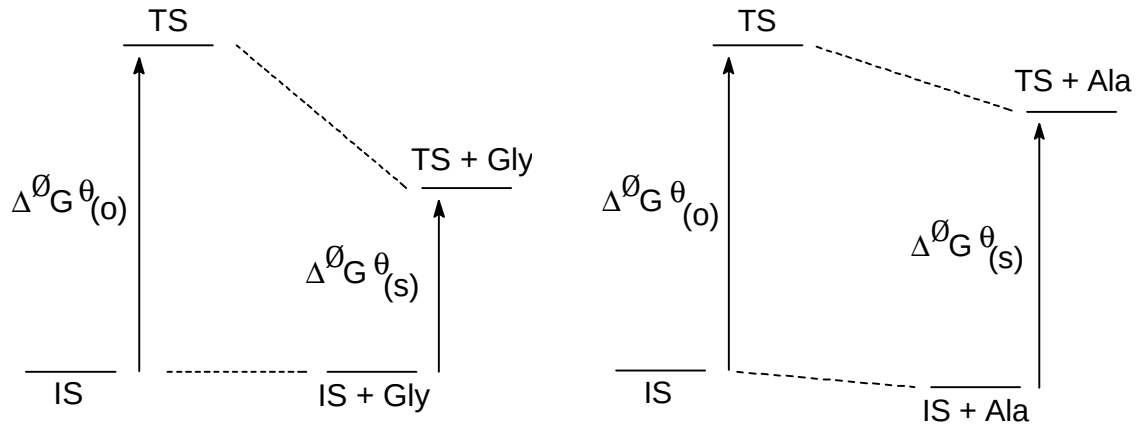
the carboxylate and the positive pole of the dipole in the activated complex in the transition state are hence considered as the dominant interactions leading to the observed rate enhancements. These interactions can occur either direct, or, more probably, via polarisation of hydration water, because direct stabilisation would imply desolvation of the carboxylate group which is energetically very unfavourable.

A comparison can now be made between the contribution of the α-CH_3 to the observed medium effect as derived from the zwitterionic α-amino acids glycine and alanine and from their amide derivatives (just for those two α-amino acids, since there is no group additivity for the apolar groups as was pointed out above):

$G(Ala)-G(Gly) = G(CH_3)_{zwitterion}$	-317 (37) J kg mol^{-2}
$G(Alaninamide)-G(Glycinamide) = G(CH_3)_{amide\ derivative}$	-86 (31) J kg mol^{-2}

From these numbers it might be concluded that the CH_3 group in the zwitterions has an interaction with the hydrophobic initial state which is more favourable than that of the CH_3 group in the amide derivatives, or in other words, has a higher apparent hydrophobicity. However, these values are misleading; the polar amide functional group is less extensively hydrated than the ionic carboxylate group, so hydrophobic interactions of the initial state of BPhT with the amide derivatives are more pronounced. The decrease in G(c) from Gly to Ala is thus primarily determined by a destruction of the carboxylate ionic hydration shell by interaction with the hydration shell of the methyl group. Therefore, the reduction in rate constant upon going from Gly to Ala is a transition state effect. The interactions of these solutes with the initial state and the activated complex in the transition state of the reaction are shown in Scheme 4.2. In

summary, it has become evident that intramolecular hydration shell overlap effects have to be taken into account in the analysis of the kinetic medium effects of the hydrolysis of BPhT. Several types of noncovalent interactions determine the kinetic effect and both initial and transition state Gibbs energies can be affected.



Scheme 4.2 *Interaction of glycine (left) and alanine (right) with the initial state and activated complex for the hydrolysis of BPhT, where* $D\,G^{q}_{(o)}$ *and* $D\,G^{q}_{(s)}$ *are the Gibbs energies of activation for the hydrolysis in pure water and in aqueous solution containing cosolutes, respectively.*

The methylation of the amino group does not induce such striking effects as does the neutralisation of the carboxylate group and shows the minor importance of the ammonium group hydration in the medium effect. One methyl group reduces G(c) from 875 to 555 J kg mol^{-2}, an effect which is similar to the change in G(c) going from Gly to α-Ala. Apparently, the site of substitution of the methyl group is unimportant in this case; or at least the different noncovalent interactions between the reactant and the isomers add up to yield the same pairwise Gibbs energy interaction parameter (the partial molar volumes for example, of alanine and *N*-methylglycine are not identical[30], indicating differences in hydration properties). A similar effect is observed in Chapter 5, where the effect of these solutes on the rate of hydrolysis of 2-(4-nitrophenoxy)tetrahydropyran are analysed.

From a study by Reading et al.[30], who determined pairwise enthalpic self-interaction coefficients for Gly and Ala and their *N*-methyl-substituted derivatives in aqueous solution (see below), it appears that the contribution of the methyl group to

Δh_{xx} (Ala) - Δh_{xx} (Gly)	-656 (±5) J kg mol^{-2}
Δh_{xx} (*N*-MeAla) - Δh_{xx} (*N*-MeGly)	-643 (±5) J kg mol^{-2}

h_{xx} is the same in the zwitterionic α-amino acids as in the *N*-methylated derivatives. In other words, the interactions of the side chain with another solute in aqueous solution is (enthalpically) not affected by the functionalisation of the ammonium group. This trend is opposite to the values reported above for the contribution of the CH_3 group to the G(c) value in the Gly and Ala zwitterions and their amide derivatives, again stressing the predominance of the carboxylate group hydration in the intermolecular interactions of α-amino acid zwitterions with the reactant.

When a second and a third methyl group are introduced at the nitrogen atom, the rate of hydrolysis of the amide is higher than that for *N*-methylglycine. This contradicts with the notion that an additional apolar group encourages hydrophobic interactions. Even the carboxylate basicity predicts the reverse effect; *i.e*. the strength of the base decreases, and a decreased polarisation of the hydration water could be anticipated (resulting in lower G(c) values). This observation can possibly be interpreted in terms of destructive overlap of the two ionic hydration shells[13-15]. Alkylation of the nitrogen atom dramatically changes its hydration, because the positive charge is screened. The ionic hydration shell of the carboxylate group is influenced by this change and apparently benefits from it.

The mutual interactions of hydration shells complicate the interpretation of the present results. Measurements of G(c) values for the hydrolysis of BPhT for a series of α-amino acids where the carboxylate is functionalised, like the α-amino acid amides, will probably reflect the side chain hydrophobicity in a more straightforward way.

In an attempt to avoid the mutual interactions of the ionic groups in the zwitterionic α-amino acids the medium effect of aqueous solutions containing ammonium cyanoacetate as a cosolute was measured. The cyano group was chosen in order to obtain a carboxylate-group basicity that approximates the pK_A of Gly. This solute is still able to speed up the hydrolysis reaction but its G(c) value is much lower than that of Gly. The carboxylate group is now able to interact without being disturbed by the hydration of the ammonium group but additional contributions to G(c) stem, of course, from interactions with the cyano group and from interactions with the ammonium group. It was observed in the previous chapter that the ammonium group itself, or more correctly, ammonium bromide, has a retarding effect on the hydrolysis of a related substituted triazole[2]. Therefore, a comparison between the two cosolutes is not completely justified. However, the observed rate reduction is considerable. This supports the assumption that the increased relative permittivity of the solution in the presence of the zwitterions plays an important role in the acceleration of the hydrolysis reaction.

A small counterion effect is observed when the ammonium ion is replaced by sodium as the counterion; the G(c) value is slightly reduced, in accord with the view that the carboxylate group dominates the medium effect. It is likely the effect of Na^+ on the water dynamics is similar to that of the ammonium ion, *i.e.* that it reduces the ability of water to find the required orientations which lead to the formation of the activated complex and subsequently to reaction. Since it is unlikely that the Na^+-ion interacts with the initial state, it may be that this transition state destabilisation is more pronounced for Na^+ than for NH_4^+, which would account for the smaller rate enhancement. On the other hand, interactions between the carboxylate and the cations should not be completely excluded, even though they are extensively hydrated and these interactions can also contribute to the observed retardations.

Finally, ammonium methanesulphonate was examined as a cosolute. Although the sulphonate anion is a very weak base and general-base catalysis by this cosolute is clearly out of the question, a positive G(c) was recorded. This result supports the hypothesis of polarisation of the water molecules in the activated complex by the anionic moiety of the cosolute and provides further evidence against general-base catalysis (*vide supra*). Unfortunately, the solubility of ammonium methanesulphonate in water is low and only measurements at low concentrations could be performed, providing a less accurate G(c) value. With the similar aim to study the effect of separation of charges, Fernández *et al.*[31] recently investigated the interactions between ammonium methanoate and several amides in water at 25°C by measuring enthalpies of dilution and compared them with those of an earlier α-amino acid study. The results are striking and suggest that when the carboxylate and ammonium groups interact with hydrophobic and peptide groups, the energetics are not significantly disturbed by the proximity of the ionic groups to each other. This is in contradiction with the explanations provided for the results in this chapter. It has to be borne in mind, however, that in their study[31] *enthalpies* of interaction are measured and that it is not the salt which is varied in hydrophobicity but the amide. Ammonium methanoate could not be used in the kinetic study due to general-base catalysis by the methanoate anion.

4.2.7 Hydrophobic α-amino acids

In contrast to the α-amino acids with aliphatic side chains, phenylalanine, which contains the hydrophobic benzylic group, shows a remarkable *retardation* of the hydrolysis of BPhT. Again intramolecular hydration shell overlap effects should be considered to understand this distinctive behaviour. Obviously, the interaction of the

carboxylate hydration shell with BPhT does not dominate the medium effect in the case of α-Phe. This is probably due to a combination of:

1) destructive overlap with either the ammonium hydration shell (due to the bulkiness of the phenyl group) or the phenyl hydration shell and
2) the hydrophobicity of the phenyl group.

Since the ionic hydration shell is partly destroyed, α-Phe mainly interacts with the reactant through hydrophobic interactions of the side chain. The remarkably large deceleration reflects the hydrophobicity of α-Phe. 3-Phenylserine also causes a retardation of the reaction rate, though less pronounced than that for α-Phe. This supports the idea that these α-amino acids mainly interact with BPhT through their side chains because a positive contribution of the hydroxyl group to the medium effect is now observed, which is expected on the basis of the structures of reactant and the activated complex. It is striking that this contribution equals the contribution of the hydroxyl group in aliphatic alcohols (see Table 4.3). Apparently, the molecular recognition process between BPhT and these zwitterionic α-amino acids is similar (in terms of Gibbs energies) to that of the non-ionic alcohols.

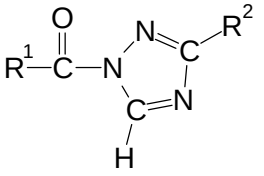
Table 4.3 *Pairwise energy group interaction parameters for the hydroxyl group with BPhT derived from a series of alcohols[28] and data from Table 4.1.*

cosolutes	**G(OH) (J kg mol^{-2})**
aliphatic alcohols	+226 (28)
α-amino acids with aromatic side chains	+230 (31)
α-amino acids with aliphatic side chains (see section 4.2.5)	-150 (50)

In order to further substantiate the dominance of the aromatic moiety in the molecular recognition process, the kinetic medium effects of phenylalaninamide and benzyl alcohol were measured. Both cosolutes retard the hydrolysis reaction as well and even more than does α-Phe, though a sufficiently reliable G(c) value could not be obtained for benzyl alcohol due to solubility constraints. This shows that a camouflaging effect of the charges, especially of the carboxylate group, on the interactions with the side chains in α-Phe and 3-phenylserine still exists.

One could assume that phenyl-phenyl stacking interactions between cosolute and reactant play a role in the observed medium effect[32,33]. Stacking interactions are also evident in proteins where aromatic side chains often occur in clusters[34]. To exclude the occurrence of phenyl-phenyl stacking and to ascribe the observed effects to hydrophobic interactions, we studied the interactions of α-Phe and α-Ala with two related substituted acyltriazoles: 1-ethanoyl-3-*tert*-butyl-1,2,4-triazole (EtBT) which lacks phenyl substituents and is slightly more hydrophobic than BPhT[35] and 1-benzoyl-

1,2,4-triazole(BT), which is less hydrophobic than BPhT[35] (see Scheme 4.3). The G(c) values are displayed in Table 4.4. Likewise the hydrolysis of BPhT, rate retardations are also obtained for the hydrolysis of EtBT, providing evidence against phenyl-phenyl stacking interactions. Interactions of the phenyl group of the α-amino acid and the heterocyclic ring of the kinetic probe are not likely, since addition of α-Phe (0.1 m) to an aqueous solution of 3-phenyl-1,2,4-triazole (0.03 m) (lacking the amide functionality and thus hydrolysis resistant) does not induce chemical shift changes in the ^{1}H-NMR spectra of both solutes.



Scheme 4.3 *The kinetic probes:*
BPhT:R^1 =R^2 =C_6H_5
EtBT: R^1 = ethyl, R^2 = *tert*-butyl
BT: R^1 = C_6H_5, R^2 = H

Table 4.4 *G(c) values[a] (in J kg mol^{-2}) for α-Ala and α-Phe with the kinetic probes.*

	probe		
cosolute	BPhT	EtBT	BT
α-alanine	558 (16)	693 (2)	478 (2)
α-phenylalanine	-709 (6)	-1483 (185)	-502 (136)

[a]*Errors in parenthesis.*

Further evidence for hydrophobic interactions playing a crucial role in the observed medium effects is provided by the results obtained with BT as a reactant. In this case the initial state stabilisation is less for α-Phe (a less negative G(c) value is obtained), which is in agreement with what is expected when hydrophobic interactions dominate the medium effect. That the G(c) value of α-Ala changes only slightly and in the opposite way, suggests a different interaction mechanism dominated by electrostatic interactions of the ionic groups. That hydrophobic interactions involving phenyl groups are strong in aqueous solutions is also evident from studies by Castronuovo *et al.*[36],who measured pairwise enthalpic interaction coefficients of various α-amino acids. Chiral recognition was observed for most of the α-amino acids as a result of a preferential configuration where the ionic groups of two α-amino acids interact with each other. However, chiral recognition was not observed for Phe, due to strong interactions between the phenyl groups. Apparently, the preferential orientation of the ionic groups could not be achieved, resulting in loss of chiral recognition. These experiments show the interplay between hydrophilic and hydrophobic interactions and the dominance of the latter in case of Phe.

To emphasise that hydrophobic interactions are dominant but not the only type of noncovalent interactions determining the kinetic medium effects in α-amino acids containing an aromatic side chain, the hydrolysis of BPhT was also measured in aqueous solutions containing α-alaninamide. If hydrophobicity of the cosolute would

be the sole determinant of the medium effect, than the difference in G(c) between α-Ala and α-alaninamide should be approximately the same as the difference in G(c) between α-Phe and α-phenylalaninamide (the same holds for G(α-Ala)-G(α-Phe) and G(α-alaninamide)-G(α-phenylalaninamide). This is clearly not the case as can be seen in Scheme 4.4, supporting the assumption that an analysis of the medium effects solely in terms of hydrophobicity of the cosolute is a fallacy; the ionic carboxylate attenuates not only the apparent hydrophobicity of aliphatic α-amino acids but also the apparent hydrophobicity of Phe. From Scheme 4.4, it can be deduced that the effect of the ionic COO^- group on the hydrophobic interactions of the phenyl group is 368 (53) J kg mol^{-2} more destructive than that for the polar $CONH_2$ group.

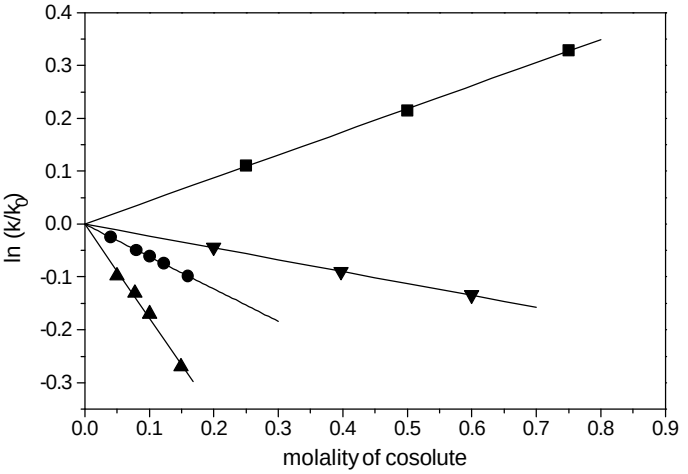
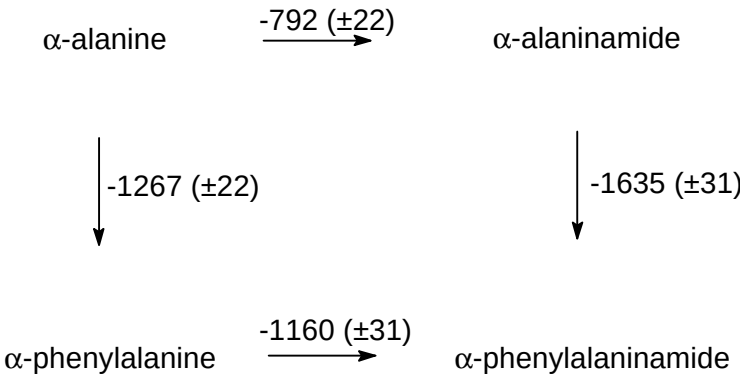


Figure 4.2 *Kinetic medium effects of alanine (■), alaninamide (▼), phenylalanine (●) and phenylalaninamide (▲) on the hydrolysis of BPhT.*



Scheme 4.4 *DG(c) values of some cosolutes in J kg mol^{-2}, which emphasises the importance of the influence of different functional groups on the interaction of the side chain substituent with BPhT.*

It has been observed previously that the amide functionality is less dominating in kinetic medium effects of BPhT[37]. The value is in the order of magnitude of the value found for the $\Delta G(CH_3)$ between the Gly and Ala zwitterions and their amide derivatives which is 231 (68) J kg mol^{-2} (see Section 4.2.6). The fact that the values are approximately the same for the phenyl and methyl group suggests that this reduction in

rate constant (upon going from the carboxylate to the amide functionality) is primarily due to reduced stabilising interactions with the transition state and not to increased stabilising hydrophobic interactions with the initial state.

In Figure 4.2, the kinetic medium effects of several rate-retarding cosolutes are visualised.

4.3 Isobaric activation parameters

To obtain additional information about the types of noncovalent interactions responsible for the observed accelerations in terms of charge stabilisation in the transition state, rate constants for the hydrolysis of BPhT have been measured as a function of temperature between approximately 18-38°C for aqueous solutions containing Gly, Ala, Val and Phe. The data were analysed in terms of the Eyring equation:

$$\ln (kh/k_BT) = - \Delta H^{\theta}/RT + \Delta S^{\theta}/R$$

In this equation, k is the rate constant, h is Planck's constant, k_B the Boltzmann constant, T the temperature (K) and R the gas constant. The slope of the plot of ln(k/T) versus 1/T gives the enthalpy of activation, ΔH^{θ}, whereas the entropy of activation, ΔS^{θ}, can be obtained from the intercept with the y-axis. The results, together with the Gibbs energies of activation, are listed in Table 4.5.

Table 4.5 *Isobaric activation parameters[a] for the hydrolysis of BPhT in aqueous mixtures containing Gly, Ala, Val and Phe*.

Solvent Composition	ΔG^{θ} (kJ mol^{-1})	ΔH^{θ} (kJ mol^{-1})	ΔS^{θ} (J mol^{-1} K^{-1})
H_2O[b]	89.65 (0.02)	45.9 (0.5)	-147 (2)
H_2O/Gly (0.5M)	88.94 (0.02)	51.6 (0.6)	-125 (2)
H_2O/Ala (0.15 M)	89.40 (0.01)	47.4 (0.3)	-141 (1)
H_2O/Val (0.5M)	89.04 (0.03)	49.7 (0.3)	-132 (1)
H_2O/Phe (0.15M)	89.78 (0.02)	52.0 (0.7)	-127 (2)

[a]Errors in parenthesis. [b]Taken from reference 38.

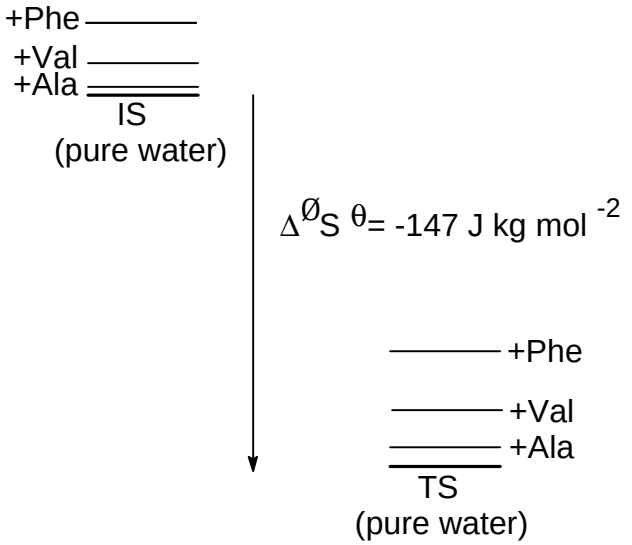
The rate enhancements observed for Gly, α-Ala and α-Val are entropy driven. The large and unfavourable negative entropy of activation for the reactions in water becomes less negative when Gly, α-Ala or α-Val are present in the reaction medium. By contrast, the enthalpy of activation becomes more unfavourable. Glycine can be considered as a dipolar ion and hydrophobic interactions with the initial state can be neglected for

reasons mentioned earlier. The change in activation parameters upon adding glycine to the aqueous solution therefore purely reflect the polar interactions of glycine with the activated complex. It is striking that in this case the acceleration is not enthalpy-driven. Presumably, the largest part of the hydrogen bonding interactions in the carboxylate hydration shell of the zwitterionic molecule is preserved during the activation process.
For α-phenylalanine, one of the cosolutes that retards the rate of the hydrolysis, the change in Gibbs energy of activation is enthalpy-driven. The entropy of activation becomes more favourable here as well, but cannot fully compensate the unfavourable change in enthalpy of activation. A microcalorimetric titration experiment in which an aqueous solution of 3-phenyl-1,2,4-triazole (0.04 m) (modelling the initial state but hydrolysis resistant) is titrated into an aqueous solution of α-phenylalanine (0.1 m) confirms the favourable enthalpic interaction between cosolute and initial state thereby supporting the enthalpy-driven retardation.

It is known that there is a large and negative enthalpic contribution to the Gibbs energy of hydration of aromatic hydrocarbons which dominates the entropic contribution[39]. This is opposite to the hydration energetics of aliphatic hydrocarbons and therefore it is assumed[39] that the mechanism of hydrophobic interactions of aromatic hydrocarbons has a different nature than that of aliphatic ones. The dominance of the enthalpy of hydration for aromatic hydrocarbons is thought to be due to hydrogen bonding interactions of the aromatic group with water, a phenomenon long discussed in the literature, but for which only recently direct evidence was provided[40]. Upon interaction of Phe with BPhT, these favourable interactions are lost (in the cosolute as well as in the reactant). However, it is anticipated that this would increase the enthalpy of the initial state more than that of the transition state, resulting in a lower enthalpy of activation. However, the enthalpy of activation is increased (see Table 4.4) and therefore it is believed that hydrogen bonding interactions between water and the aromatic groups are not dominating the activation enthalpy.

As for hydrophobic interactions, they are characterised by large entropy increases[41]. When hydrophobic interactions between the larger side chains and the initial state come into play, it is anticipated that the entropy of activation becomes more negative going from Ala→Val→Phe, because the initial state (more hydrophobic) is raised more in entropy than is the transition state. However, the opposite trend is observed. When sticking to the idea of an initial state (IS) which is raised in entropy due to hydrophobic interactions, this implies an even larger increase in entropy of the transition state (TS). This idea is illustrated in Scheme 4.5; the entropy of the initial state is assumed to be increased by hydrophobic interactions, and more so by the more hydrophobic α-amino acid. However, the entropy of the transition state has to be decreased more dramatically to account for the observed trend in the values for ΔS^{θ}

when hydrophobic interactions are dominant, which would imply increased hydrophobic interactions with the less hydrophobic activated complex in the transition state, which is a contradiction. Thus, though the activation parameters shed some light on the thermodynamics of the interaction processes, it is clear that the available data are insufficient to assess the contributions of the different types of noncovalent interactions with the IS and the activated complex. The nature of the dominant interactions in these series is variable. At present it is not feasible to unravel the details of the thermodynamic interplay of the different interactions between zwitterionic amino acids and amides in aqueous solution. Obviously, a description of pairwise solute-solute interactions in terms of one single interaction mechanism is not warranted.



Scheme 4.5 *$D\ S^{q}$ for the hydrolysis of BPhT in water and in aqueous solutions containing Ala, Val and Phe, based on hydrophobic interactions.*

4.4 Results in retrospect; Consequences of intramolecular hydration shell overlap effects on intermolecular interactions

In this chapter, the kinetic medium effects of aqueous α-amino acids solutions on the water-catalysed hydrolysis of an activated amide were investigated. All α-amino acids, except α-phenylalanine and 3-phenylserine, increase the reaction rate. It is the first time that substantial rate enhancements are observed for kinetic medium effects on the hydrolysis of BPhT. Strong evidence was obtained that general-base catalysis by the carboxylate group is not the origin of this rate increase. However, from measurements using several α-amino acid derivatives, it nevertheless appears that the carboxylate group is the main determinant in the observed rate effects.

It is plausible that the rate enhancements are a transition state effect, brought about primarily by an electrostatic stabilisation via polarisation of hydration water due to the electrostatic field created by the zwitterions (actually a salt effect), or more precisely, by the carboxylate groups. A molecular dynamics study of the properties of water around methylammonium and acetate ions[42] showed that there is some increase in solvent polarisation around the $-CO_2^-$ group (and a decrease of the solvent polarisation around the $-NH_3^+$ group). It is indicated[42] that this increase is consistent with the large partial charges on the carboxylate and its favourable geometry and charge distribution to provide H-bonding opportunities more favourable than those in the bulk. This can be an important factor in the interactions of the hydration shell of the carboxylate group with that of the dipolar activated complex in the transition state of BPhT.The electrostatic field generated by the charged groups can increase:

1) the electrophilicity of the carbonyl C-atom in the TS of BPhT (Figure 4.4*a*);
2) the polarisation of the 'second' water molecule in the TS which acts as a general base (*i.e*. increase the kinetic basicity of water) (Figure 4.4*b*).

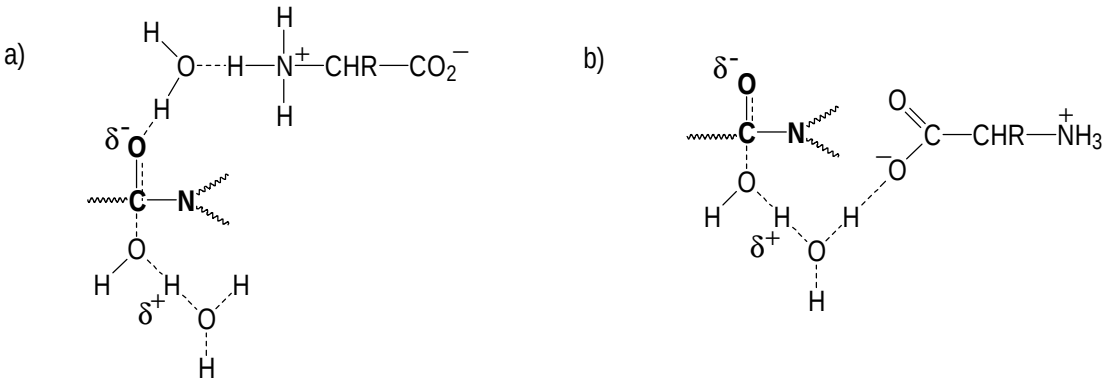


Figure 4.4 *Stabilisation of the transition state of BPhT by increasing (a) the electrophilicity of the carbonyl C-atom and (b) the kinetic basicity of water through polarisation of water molecules.*

Both effects will cause a stabilisation of the transition state and can account for the observed rate enhancements in the hydrolysis of BPhT. Since the experimental results indicate that the ammonium group does not contribute to the rate enhancements, it is presumed that either the electrostatic interactions of the charged ammonium group with the polarised carbonyl group of the activated complex in the transition state do not take place or are more than counteracted for by the restrictive effect of the ammonium group on the required water orientation in the TS (destabilising). The latter is an acceptable assumption in view of the observations described in Chapter 3, where the ammonium group contributes negatively to the medium effects on the hydrolysis of BT.

Another effect of the interaction of the carboxylate with the activated complex via the solvent water needs to be considered. The water around the carboxylate group of the acetate ion is not only more polarised, but both solvent-solvent and solute-solvent components of the binding energy are more favourable than in the bulk (as appeared from the same molecular dynamics simulation study mentioned in the previous paragraph[42]. For the other solvation shells (those of the ionic group (ammonium) and apolar groups (methyl), the binding energy of water remains about the same as in the bulk. Because of this strong hydrogen bonding there is a definite propensity for proton tunnelling[43]. Recently, it was shown that quantum mechanical tunnelling plays a role in the proton transfer (*i.e*. rate limiting step) of the water-catalysed hydrolysis of *p*-methoxyphenyl dichloroacetate[44], a reaction with the same mechanism as the hydrolysis of BPhT**.** Therefore, it is likely that proton tunnelling plays a role in the hydrolysis of the activated amide BPhT as well. Possibly, the quantum dynamical nature of the proton transfer is more pronounced in solutions containing groups which strengthen the water-water interactions, like the carboxylate group. This would then result in a faster proton transfer, and a concomitant increase of the reaction rate.

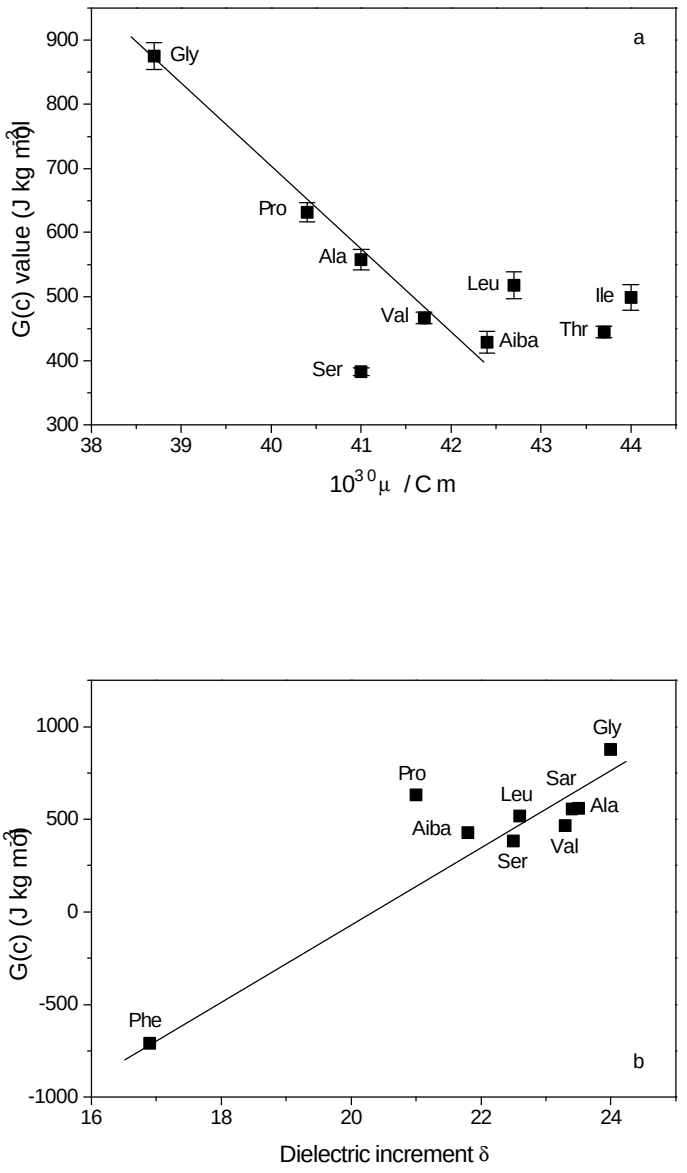


Figure 4.5 *Plots of the kinetic medium effects (expressed in G(c)) vs. (a) the solute dipole moment and (b) dielectric increment (error bars fall within data points). Data taken form ref. 45 and 46, respectively.*

Assuming that the polarisation of the solvent is an important factor in the rate enhancements, a relation between the G(c) value and the dipole moment of the α-amino

acid can be anticipated. As is shown in Figure 4.5*a*, such a trend may exist but it is unsure due to the limited data available.

In section 4.2.1 it was also mentioned that α-amino acids increase the relative permittivity of water considerably, which can contribute to the observed rate accelerations. This is also consistent with the G(c) values observed for diglycine (1488 (61) J kg mol^{-2}) and triglycine (1420 (36) J kg mol^{-2}), which have considerably larger dielectrical increments than the α-amino acids[7]. Though this increase may explain the observation of enhanced rates, dielectrical increments of amino acid solutions are very similar and their values do not appear to explain the kinetic trends (Figure 4.5*b*). In addition it has to be kept in mind that the relative permittivity is a macroscopic property of the solvent. Two difficulties then arise:

1) The solvent is considered as a continuum up to the ion.
2) The relative permittivity of the solventis taken as a constant value, the bulk value.

However, near (zwitter)ions the *local* value of ε can change very rapidly[47]. Without knowledge about the exact local relative permittivity of the solvent in the direct neighbourhood of the zwitterions, more value should be attached to Fig 4.5*a* than to Fig 4.5*b*.

The carboxylate hydration is subject to influences from other internal hydration shells. α-Amino acids with side chains weaken the accelerating effect of the carboxylate moiety. However, the effects of a methyl, ethyl or *n*-propyl side chain are not distinguished by the amide. So, neither the hydrophobicity of the side chain nor the additivity of apolar substituents is reflected by the kinetic medium effects. These results are consequences of the large and masking effects of the hydrated ionic groups which prevent the side chains to interact via hydrophobic interactions with the activated amide. The carboxylate group plays the principal part in this masking effect. With the alkyl ammonium bromides as cosolutes (Chapter 3) at least differences in kinetic medium effects were observed. It is recognised by other authors that the effect of the COO^- group on hydrophobic interactions is more destructive than that of the NH_3^+ group[48].

A quaternary α-carbon atom, as in Aiba, however, has a distinctive effect. Aiba reduces the rate of hydrolysis more than its mono-substituted isomer. Also Val, with the *i*-propyl side chain reduces the rate more than nVal (*n*-propyl) does. All these effects can be explained in terms of reduced carboxylate hydration. It is expected that α-amino acids with longer side chains will show a more dramatic decrease of the carboxylate-induced acceleration. However, their low solubility does not allow such a study. Moreover, bulk hydrophobic interactions may come into play, due to cosolute aggregation.

The introduction of a hydroxyl group showed an unexpected relative deceleration, but this can be explained in terms of intramolecular hydration shell overlap as well. The fact that the introduction of a hydroxyl group in Phe causes a relative acceleration supports the idea that in case of α-amino acids with aromatic side chains the medium effect is dominated by hydrophobic interactions, whereas in α-amino acids lacking an aromatic group, ionic interactions play the major role.

The present study suggests that intramolecular destructive hydration shell overlap effects should be taken into account in understanding intermolecular interactions and conformational preferences of biomolecules, including protein folding.

4.5 Experimental procedures

Materials. All α-amino acids and derivatives were used as supplied by Janssen Chimica, Fluka or Sigma. The kinetic probes were available. Their syntheses have been described in the literature[9].

Ammonium cyanoacetate was prepared by leading gaseous ammonia through a solution of cyanoacetic acid in dry diethyl ether. The white solid that was formed was purified by extraction in a Soxhlet apparatus with dry ether and dried afterwards in vacuo. Sodium cyanoacetate was prepared by adding an equivalent amount of sodium ethanoate to a solution of cyanoacetic acid in absolute ethanol. The solution was heated to dissolve the salt and subsequently filtered. The white solid that crystallised upon cooling was dried in vacuo.

Kinetic measurements. Kinetic experiments were carried out according to the procedures described in Section 2.7. For the probes kinetic probes the hydrolysis reactions were monitored by following the change in absorbance at appropriate wavelengths. In the case of BPhT care was taken that the absorption did not exceed 0.5 because of the low solubility of BPhT.

For the determination of isobaric activation parameters, rate constants were measured at eight temperatures in the range of 16-38°C. Plots of ln(k/T) versus 1/T were perfectly linear, indicating that in this temperature range ΔH^{θ} is independent of temperature. ΔH^{θ} was calculated by linear regression. The ΔS^{θ} was calculated from ΔH^{θ} and ΔG^{θ} at 25°C.

For the solvent kinetic isotope effects the measurements in D_2O were performed in solutions which were adjusted to pD 4 with DCl. No deuterated glycine was used which might explain the slightly lower solvent deuterium isotope effect for the latter, as a result of some H/D exchange.

Microcalorimetry. An Omega Titration Microcalorimeter (Microcal, Northampton, MA, USA) was used to measure changes in enthalpy accompanying the computer-controlled titration of an aqueous solution of 3-phenyl-1,2,4-triazole (0.04 m) to an aqueous solution of α-Phe (0.1 m) at 30°C and pH 4. The changes in enthalpy due to the dilution of 3-phenyl-1,2,4-triazole were subtracted from these data.

^{1}H-NMR. ^{1}H-NMR spectra were recorded on a Varian VXR-300 spectrometer at 30°C. Solutions of α-Phe and 3-phenyl-1,2,4-triazole (concentrations of 0.10 molal and 0.03 molal, respectively) were prepared in D_2O, and adjusted to pD 4 with a DCl solution. H_2O was used as internal standard. Changes in chemical shifts were smaller than 0.02 ppm.

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CHAPTER 5

Effects of Anionic α-Amino Acid Hydration on Noncovalent Interactions

5.1 Introduction

In the previous chapter the dominant effects of the carboxylate and the ammonium group hydration in zwitterionic α-amino acids on their noncovalent interactions with a hydrolytic organic compound have been discussed.

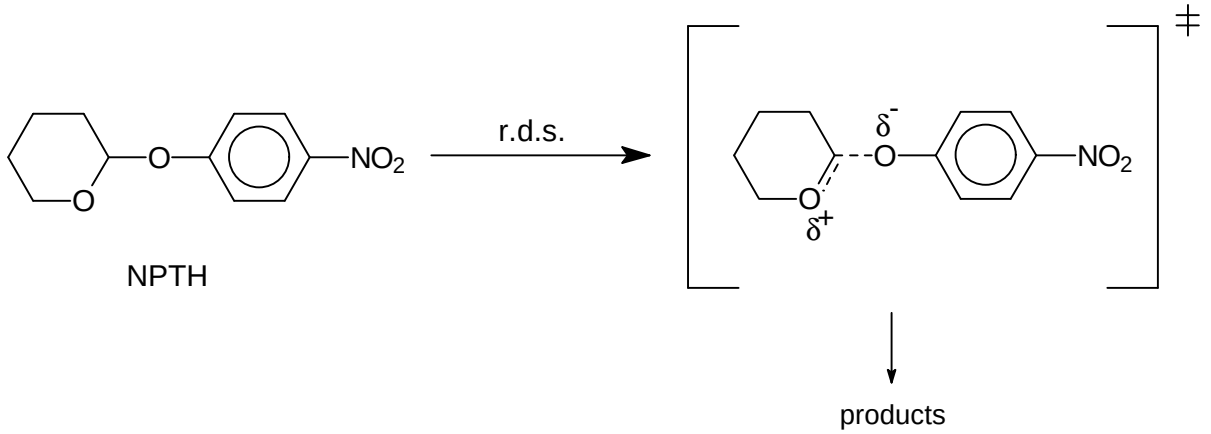
Since the ionic state of α-amino acids is tuneable by altering the pH of the solution, this gives the opportunity of investigating the hydration characteristics of cationic α-amino acids (at low pH) and anionic α-amino acids (at high pH). Neutralisation of one of the charged groups of the α-amino acid zwitterion causes a decrease in hydration of that group. For example, the partial molar volume in aqueous solution increases by 6 cm^3 mol^{-1} when the ammonium functionality is neutralised to an amino functionality and 2 cm^3 mol^{-1} when the carboxylate group is neutralised to a carboxyl group (due to a decrease in solvent electrostriction)[1]. It is therefore anticipated that more insight into intermolecular interactions involving α-amino acid side chains can be obtained when one of the polar termini is less extensively hydrated, because overlap of the hydration shells in the intermolecular interaction process will be reduced.

When these effects are investigated by means of kinetic solvent effects as was done in the previous three chapters, the hydrolysis of substituted 1-acyl-1,2,4-triazoles is not a sensible choice as a model reaction, since it exhibits acid and base catalysis at low and high pH values, respectively, and second-order rate constants would need to be determined in addition to k_0 (rate constant for the water-catalysed reaction). Therefore, a hydrolysis reaction with (pseudo-)first-order kinetics at low or high pH would be required. As the solubility in water of α-amino acids decreases considerably at low pH, a hydrolysis reaction with (pseudo-)first-order kinetics at high pH-values is preferred.

5.2 Kinetic solvent effects on the hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran

A hydrolysis reaction which fulfils the requirement of exhibiting first-order kinetics at high pH is the unimolecular solvolysis of 2-(4-nitrophenoxy)tetrahydropyran (NPTH)

(Scheme 5.1). At low pH values (pH<4) this reaction is subject to general acid catalysis[2]; at higher pH values the reaction is pH-independent[3,4]. At pH 11 the formation of the 4-nitrophenoxide anion can be conveniently monitored with Vis-spectroscopy. Clearly, the reaction mechanism is different from the neutral hydrolysis of the substituted 1-acyl-1,2,4-triazoles, which are water-catalysed. The



Scheme 5.1 *Reaction mechanism of the unimolecular hydrolysis of 2-(4-nitrophenoxy)tetrahydropyran (NPTH).*

unimolecular breakage of the C-O bond via a dipolar and late transition state determines the rate of the hydrolytic reaction. Bond breakage is far advanced, if not complete, in the transition state of this S_N1 reaction[3]. Since the activated complex in the transition state is much more polar than the initial state, the solvent interacts differently with these two states and this difference is reflected in the rate constants for the hydrolysis. The noncovalent interactions of the solvent with the reactant will include H-bonding, dipole-dipole, London dispersion interactions, and particularly in dilute aqueous solutions, hydrophobic interactions with apolar cosolutes.

Kinetic solvent effects on the hydrolysis of NPTH have been investigated previously[5]. In that particular study the kinetics of the hydrolysis reaction were determined in binary mixtures of water and substantial amounts of organic cosolvents, to avoid complications of hydrophobic interactions in order to obtain more insight into other types of noncovalent interactions involving NPTH and apolar cosolvents.

In this chapter the aim is to establish the extent of *pairwise* hydrophobic interactions of the cosolutes (anionic α-amino acids) with NPTH and its activated complex in the transition states of the hydrolysis reaction and, consequently, highly diluted aqueous solutions have been studied.

To quantify these pairwise interactions the thermodynamic approach to evaluate rate constants that was used in Chapters 2,3 and 4 was again applied to

the kinetic solvent effects on the hydrolysis of NPTH. The difference in Gibbs energy of pairwise interactions in the initial and transition states (expressed as G(c)) is related to the rate constants as described in Chapter 2. However, since water is not involved in the rate determining step, the effect of the cosolutes on the reactivity of water needs no consideration and the second term in the equation can be omitted, leading to the following equation, which quantitatively describes the kinetic solvent effects on the hydrolysis of 2-(4-nitrophenoxy)tetrahydropyran:

$$\ln(k_m/k_{m=0}) = 2G(c)m/RT$$

First, the effects of short-chain primary alcohols was investigated to check the applicability of this equation, *i.e.* the quantitative description in terms of the solute-solute pairwise interactions. The SWAG-approach for the additivity of functional groups was tested as well. Second, a series of α-amino acids was studied together with some methylated glycines. Finally some oligoglycines and two other dipeptides have been investigated. In these cosolutes the charged and polar termini are separated to a longer distance and the interactions of the cosolutes with the kinetic probe are anticipated to be less influenced by overlap of the hydration shells of these moieties. All measurements have been carried out at 40°C, because the reactivity of NPTH is rather low at room temperature.

In the last section of this chapter, the kinetic medium effects will be correlated with available literature data which either reflect solution properties (like partial molar properties, dynamic hydration numbers, some hydrophobicity scales) or intrinsic properties (like polarisabilities and dipole moments) of α-amino acids in order to put the results into a broader perspective.

5.2.1 Dilute aqueous alcohol solutions

Rate constants for the hydrolysis of NPTH in mixtures of water with MeOH, EtOH, 1-PrOH, 1-BuOH and 2-BuOH, were determined at several molalities of alcohol up to about 1.5 molal, except for 1-BuOH which was measured only up to 0.75 molal due to solubility constraints. All alcohols retarded the rate of hydrolysis as was anticipated on the basis of their hydrophobic nature; hydrophobic interactions in the apolar initial state of the acetal hydrolysis will be more pronounced than in the dipolar transition state. Plots of $\ln(k_m/k_{m=0})$ versus the molality of alcohol gave excellent linear correlations (Figure 5.1). The G(c) values were obtained from the slopes of these plots (Table 5. 1). For the analysis of the results in terms of the

Table 5.1 *Pairwise Gibbs energy interaction parameters for primary alcohols and relative retardations at 1 molal concentration*.

Alcohol	G(c) (J kg mol^{-2})[a]	ln ($k_{m=1}/k_{m=0}$)
MeOH	-179 (8)	-0.14
EtOH	-299 (11)	-0.24
1-PrOH	-422 (14)	-0.34
1-BuOH	-621 (7)	-0.50
2-BuOH	-601 (20)	-0.49

[a]Errors in parenthesis

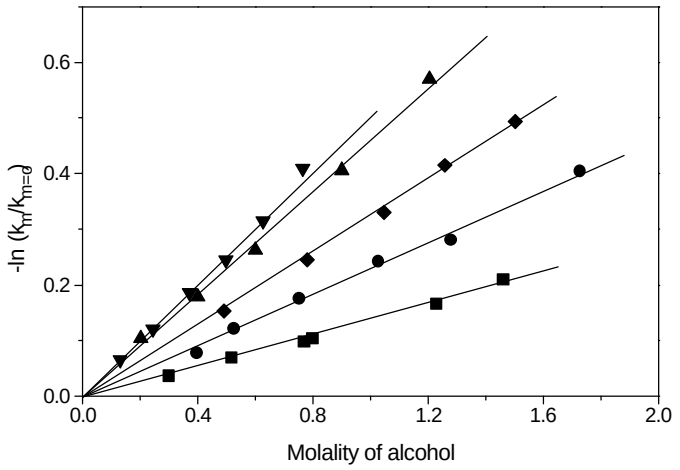


Figure 5.1 *Effects of methanol (■), ethanol (●), n-propanol (◆), 2-butanol (▲) and n-butanol (▼) on the hydrolysis of NPTH.*

SWAG-approach[6], a plot of these G(c) values versus the number of CH-groups (n(CH)) in the cosolute was constructed (Figure 5.2). This figure shows a linear relationship which confirms the additivity of the pairwise Gibbs energy contribution of the CH-groups in the cosolute (where 3G(CH) = 1½G(CH_2) = G(CH_3)[7]) to the medium effect (see also Chapter 1). Thus, pairwise *group* interaction parameters can be obtained *i.e.* G(CH) from the slope of Figure 5.2 and G(OH) from the intercept with the y-axis (n(CH) = 0) in the same figure, which are -61 (9) J kg mol^{-2} and 4 (5) J kg mol^{-2}, respectively. In other words, the CH-group has a negative contribution to the observed rate effect, which can be explained by dominating stabilising hydrophobic interactions between the cosolute and the initial state. Apparently, the OH-group of the alcohols has a similar interaction with the reactant and the activated complex, because its contribution to the overall solvent effect is negligible. This contrasts with the effects of alcohols on the hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole[8]. Although for these activated amides the same G(CH) was obtained within experimental error (-68 J kg mol^{-2}), a much larger positive value for G(OH), 226 J kg mol^{-2}, was calculated. This difference may be rationalised in terms of the reaction mechanism of the amide hydrolysis, which involves two water molecules in the transition state[9]. The Gibbs energies of

interaction of the alcoholic OH-group with the reactant and the activated complex will therefore differ substantially from those of the acetal hydrolysis, as there are favourable interactions between the cosolute OH-group and the OH-groups of the activated complex in the transition state. Since the G(CH) for both reactions is the same, it seems justified to state that the intermolecular interactions between the CH-groups and the two reactants in the two studies proceed via the same molecular recognition process and are of hydrophobic nature.

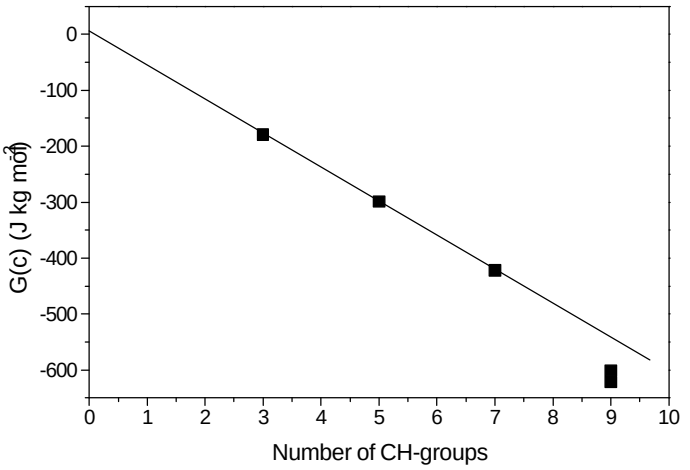


Figure 5.2 *Pairwise Gibbs energy interaction parameters for the hydrolysis of NPTH as a function of the number of CH-groups in the alcohol.*

5.2.2 Dilute aqueous α-amino acid solutions

Now that the thermodynamic approach for the evaluation of solvent effects in dilute aqueous solutions has proven its validity for the hydrolysis of NPTH, attention can be turned towards the cosolutes of interest, the anionic α-amino acids.

As was mentioned in the introduction, the effects of α-amino acids at pH 11 on the hydrolysis of NPTH are the final result of effects caused by the $-CO_2^-$, $-NH_2$ (on average 5.5% is in the protonated state) and -R (side chain) moieties, where it is anticipated that the effect of R is more dominant, possibly through hydrophobic interactions with the reactant.

Two general observations do appear immediately from the kinetic measurements. The first is the retardation of the hydrolysis of NPTH in aqueous α-amino acid solutions (with the exception of Gly whose effect on the reaction rate is negligible). Presumably, hydrophobic interactions between the α-amino acid side chains and the initial state of the reaction do indeed govern the kinetic medium effect. It seems justified to conclude that anionic amino acids behave primarily as hydrophobic solutes and zwitterionic amino acids as hydrophilic solutes, except for Gly, which behaves as a hydrophilic solute in both studies and Phe, which behaves

as a hydrophobic solute in both studies. Secondly, the effects of the α-amino acids on the kinetics of the hydrolysis of NPTH do not exhibit linear correlations between ln ($k_m/k_{m=0}$) and the molality of the α-amino acid, but instead show a curvature which increases with increasing molality and hydrophobicity of the α-amino acid. Therefore, the quantitative description of the pairwise interactions as used to analyse the results for the alcohols cannot be applied to the α-amino acids and consequently no G(c) values can be derived. Maybe higher-order interactions come into play but the results have not been fitted to a higher-order equation. Instead, to quantify the relative rate retardations induced by the additives, the natural logarithms of the relative rates at 1 molal of α-amino acid were plotted against the number of CH-groups in the α-amino acid side chain. At this concentration the rate differences between the different cosolutes are quite pronounced. The number of CH-groups in Gly was assigned 0, since it has no side chain, α-Ala has 3 CH-groups and so on. The number of (CH)-groups in β-Ala was arbitrarily set at 2. The results are displayed in Table 5.2 and shown graphically in Figure 5.3.

Table 5.2 *Natural logarithm of the relative reaction rate of NPTH in water containing 1 molal of amino acid.*

Cosolute	**ln ($k_{m=1}/k_{m=0}$)**	**Cosolute**	**ln ($k_{m=1}/k_{m=0}$)**
Gly	+0.010	Ile	-0.277
β-Ala	-0.032	Leu	-0.331
α-Ala	-0.066	Leu[1)]	-0.142
Thr	-0.096	nLeu[1)]	-0.164
Aba	-0.115	Phe[1)]	-0.315
Pro	-0.140	β-Phenylserine[1)]	-0.230
Aiba	-0.098	Lys	-0.174
Val	-0.180	NaAc	+0.035
nVal	-0.229		

[1)]Values at 0.5 molal of added amino acid

For comparison, the corresponding values for the alcohols at 1 molal concentration have also been included in this figure. Clearly, the effect caused by the α-amino acids is smaller than that caused by alcohols with the same number of CH-groups. The free amino group and the carboxylate group are extensively hydrated at pH 11 and presumably camouflage the interactions of the apolar groups with the reactant more than does a hydroxyl group. The linear relationship (with a break at n(CH) = 6) observed in Figure 5.3 can be interpreted in terms of CH-group additivity within the amino acid series. It is remarkable to see that proline (an imino acid with

consequently different H-bonding abilities and a cyclic structure) fits into the range though it is on the breakpoint of the two lines. The fact that threonine fits in is anticipated because the contribution of the OH-group to the kinetic solvent effect is negligible. Serine therefore is expected to show similar behaviour but its effect has not been measured. In terms of the additivity rules, structural isomers should exhibit similar rate effects, because they have the same number of CH-groups, but it is found that when isomers of the α- amino acids with longer alkyl chains are considered, they exert different effects on the rate of hydrolysis. This observation, as well as the breakpoint in the linear correlation (Fig. 5.3) can be rationalised as follows. The additivity of the (CH)-contribution to the solvent effect for n(CH) ≤ 6 is a "masked" contribution. These CH-groups are all situated *within* the hydrophilic hydration spheres of the $-NH_2$ and $-CO_2^-$ groups. The hydrophobic hydration shells of the CH-moieties are therefore badly developed and their retarding effect on the hydrolysis is diminished. Consequently, they possess a lower apparent hydrophobicity. When n(CH) > 6, the additional CH-groups are presumably situated *outside* the hydration spheres of the polar groups and now their hydrophobicity equals the hydrophobicity of the CH-group in the alcohols. Even this larger CH-group contribution (at n(CH) > 6) to the solvent effect is probably still a "masked" contribution; a more critical look at Figure 5.2 reveals that the 'perfect' additivity of the CH-groups in alcohols holds up to n(CH) = 7 and that for n(CH) = 9 a definite deviation from additivity develops.

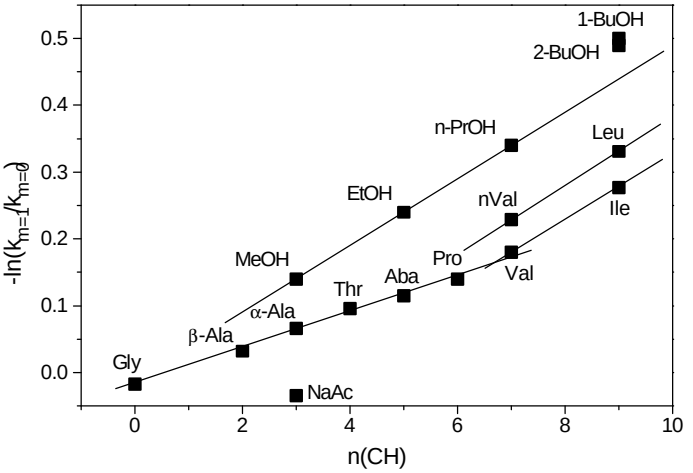


Figure 5.3 *Relationship between the number of CH groups (n(CH)) in the amino acid side chain and their effects on the rate of hydrolysis of NPTH at 40°C and pH 11 at 1 molal of amino acid. As a comparison, retardations for alcohols at 1 molal are also plotted versus the number of CH-groups.*

Previous kinetic studies involving hydrolysis reactions of substituted 1-acyl-1,2,4-triazoles also indicate that the hydration of ionic and polar groups reduce the apparent hydrophobicity of neighbouring methylene groups. These studies include the effects of α-amino acids (Chapter 4)[10], sodium *n*-alkyl sulfates[11], alkylammonium bromides (Chapter 3)[12] and *N*-alkyl-2-pyrrolidinones (Chapter 2)[13]. In the first three

of these studies the hydrophobicity of the alkyl chains is completely camouflaged by the ionic hydration shell(s), whereas in the latter study additivity of apolar group interactions is observed within the polar amide hydration shell for the first three consecutive carbon atoms in the alkyl substituent. Clearly the polar amide hydration shell is not as overwhelming as the ionic hydration of the ammonium, carboxylate and sulfate groups. In view of these studies it is surprising that the different anionic α-amino acids show measurable differences at all in the hydrolysis of NPTH.

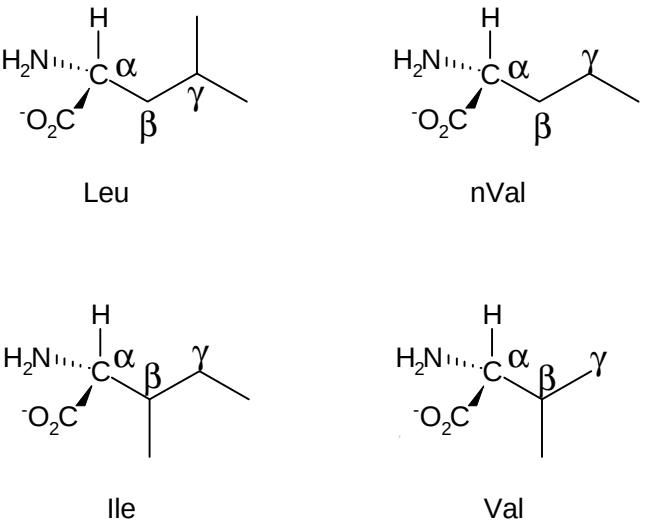
The importance of the effect of ionic hydration of α-amino acids and α-amino acid derivatives on the apparent hydrophobicity of nearby apolar groups leading to non-additive behaviour is not a new concept and has been ascertained by other investigators by measuring hydration properties by different techniques. These include measurements of apparent and partial molar volumes[14,15,16,17,18,19], partial molar heat capacities[20,21], partial molar expansibilities and adiabatic compressibilities[18,22] of amino acids; measurements of heats of dilution by microcalorimetry to obtain pairwise enthalpic heterotactic interaction coefficients of solutions containing α-amino acids and alkalimetal halides[23], and pairwise enthalpic self-interaction coefficients for α-amino acids[24], *N*-acetyl amino acids[25] and α,ω-amino acids[26]; measurements of acoustic nonlinearity parameters[27,28]; measurements of dynamic hydration numbers of α-amino acids and amines via ^{17}O-NMR[29,30]; calculations of the interaction energies of the acetate and methylammonium ions via a molecular dynamics simulation[31] and measurements on the thermodynamics of protein denaturation in the presence of α-amino acids by differential scanning calorimetry[32].

Moreover, in a number of these studies[14,18,19,22,23,29,32] it is observed that the ionic hydration shells exert their destructive effect on the hydrophobic hydration shell up to the γ-C atom, which is in agreement with the kinetic findings just presented. From these results the 'thickness' of the ionic hydration shell can be approximated to 3-4 Å. In fact Pettitt and Karplus[33] calculated that the interaction between >NH and >CH– acts within 3 Å in spacing. When the effective diameter of a water molecule is taken as 2.75 Å[34], this corresponds to 1-1½ layers of water molecules. Thus, even for solute-solvent interactions like those between water and charged atomic groups, these interactions do not seem to perturb the structure of the bulk solvent to a long range.

The results for the isomers (Val/nVal and Ile/Leu) can also be explained in terms of extension of the ionic hydration shell. Whereas the side chains of Val and Ile are branched on the β carbon atom, Leu is branched on the γ-atom and nVal has a chain elongation on γ-C (see Scheme 5.2). Introduction of a methyl group on the β-C atom represents an extension of the alkyl chain *within* the hydration spheres of

the polar groups, whereas introduction of a methyl group on γ-C is an extension of the alkyl chain *outside* the influence spheres of the polar groups

In the literature there are a few examples of studies in which branching of the side chain near α-C leads to decreased hydrophobicity of this side chain. Miyagishi *et al*.[35] measured critical micelle concentrations (cmc's) for *N*-dodecanoyl amino acids and observed lower CMC values (higher side chain hydrophobicities) for the surfactants with nVal and Leu side chains as compared to those with Val and Ile side chains, respectively. In an NMR-study by Okouchi *et al.*[29] the spin-lattice relaxation times, T_1, of natural abundance $H_2{}^{17}O$ of aqueous solutions of amines were measured. From these data, dynamic hydration numbers (n_{DHN}) have been calculated, which reflect the mobility of the water molecules in the hydration shell of the solute. The series of the butylamine isomers show that the hydrophobic hydration shell of the alkyl chain in *i*-butylamine (comparable to Leu) is better developed (lower n_{DHN}) than that of *s*-butylamine (comparable to Ile). It is remarkable that the same authors found opposite trends for zwitterionic α-amino acids six years prior to this study[30]; lower n_{DHN} are observed for Val and Ile compared to nVal and Leu, suggesting more dynamical hydration water for the latter two solutes. Maybe this contrast has to be explained in view of the zwitterionic nature of the solutes. The value of n_{DHN} reflects the mobility of water molecules in the apolar hydration spheres as well as in the ionic hydration spheres. As was discussed in Chapter 4, mutual interactions between these hydration layers can lead to unexpected apparent side chain hydrophobicities[10]. Sarvazyan *et al*.[27] also observed the dependence of α-amino acid-water interactions on branching at the β- and γ-positions in nVal/Val and Leu/Ile by ultrasonic velocimetric measurements. Recently, Suzuki *et al*.[36] studied the hydrophobic hydration of amino acid aqueous solutions by a microwave dielectric method with which the number of rotationally restrained water molecules were obtained. There appeared to be more restrained water molecules on nLeu and nVal side chains than on the Ile and Val side chains, indicating a more completely



Scheme 5.2 *Branching of the long-chain α-amino acids as a key to the explanation of their kinetic effects on the hydrolysis of NPTH.*

developed hydrophobic hydration shells for nLeu and nVal. Interestingly, the results obtained for Leu and Ile were similar.

By connecting the data points obtained for nVal and Ile (not shown in Figure 5.3) which represents the introduction of a CH_3-group *within* the hydration layers of the polar groups the obtained line runs parallel to the line connecting the data for the α-amino acids with n(CH) ≤ 6. This is anticipated and consistent with the ideas postulated above. Because the solubility of nLeu at pH 11 is insufficient to measure its kinetic effect at 1 molal, both nLeu and Leu were measured at 0.5 molal. It was anticipated that these isomers have similar effects on the reaction rate. In fact nLeu retards the reaction slightly more than does Leu (Table 5.2). This indicates that the polar hydration spheres extend to even larger distances, but the availability and solubility of longer-chain α-amino acids are limited and prevent a more detailed investigation.

The retardation caused by Aiba (α-amino*iso*butyric acid), an isomer of Aba (α-aminobutyric acid) and the only amino acid which has two α-C substituents, is slightly less than anticipated on the basis of additivity. In Chapter 4 Aiba also showed a deviant behaviour from that of the common α-amino acids[10]. However, Aiba showed a higher apparent hydrophobicity than Aba in its interaction with 1-benzoyl-3-phenyl-1,2,4-triazole whereas in the interaction with 2-(4-nitrophenoxy)tetrahydropyran Aiba has a lower apparent hydrophobicity. This seems to indicate that the details of the molecular recognition process for Aiba by the reactant are different in the two studies, which is probably caused by probe-specific interactions.

The retardation of the hydrolysis of NPTH caused by lysine (Table 5.2) is not easy to explain in terms of additivity of functional group interactions due to the additional polar group in the side chain. The pK_a of the Lys side chain amino group is 10.53. Therefore, about one third of those amino groups are protonated at pH 11 and they will be differently hydrated. It is obvious that the retardation caused by Lys is less than expected on the basis of the number of CH-groups in the side chain (which is 8).This is thought to be due to a camouflaging effect of the side chain polar group (either neutral or protonated) on neighbouring CH_2-group(s).

Sodium acetate slightly accelerates the reaction. This would lead to the conclusion that the amino group in the α-amino acids exerts a slightly decelerating effect. However, this conclusion is uncertain as we have no information about the mutual interactions of the incompatible hydration spheres of $-NH_2$ and $-CO_2^-$, which at least partly overlap[1,37,38,39]. From the absence of a significant rate effect caused by Gly we conclude that the combined effects of the two polar groups in the amino acid molecule cancel (the central CH_2-group is not available for any type of interaction

with the substrate). On the other hand, from the extrapolation of the α-amino acids Leu→ nVal→ Aba to n(CH) = 0, which is more fair because it deals with the additivity of methylene groups that are not influenced by ionic hydration, a contribution of (NH_2 + CO_2^-) is obtained, which is significantly positive. This pattern may be explained by favourable electrostatic interactions between the carboxylate and amino group of the amino acid with the polar activated complex of the reaction. This argumentation would imply that not only hydrophilic hydration reduces hydrophobic interactions of nearby methylene groups but that the effect is mutual: hydrophobic groups influence hydrophilic hydration as well.

The kinetic effects exerted by phenylalanine and β-phenylserine are particularly striking. These cosolutes have low solubilities in water and so their kinetic medium effects were measured at 0.5 molal (Table 5.2). Even at these low molalities it is obvious that these cosolutes are engaged in a different molecular interaction mechanism with the reactant than the aliphatic α-amino acid chains. They retard the hydrolysis to a significantly larger extent than anticipated on the basis of the additivity as shown in Figure 5.3. The distinctive behaviour of these α-amino acids has also been observed for the hydrolysis of 1-benzoyl-3-phenyl-1,2,4,-triazole. Since aromatic π-stacking has been excluded (Chapter 4)[40], the effect has been attributed to the pronounced hydrophobicity of these cosolutes.

5.2.3 Dilute aqueous solutions of glycine derivatives

Interesting results (Table 5.3) were obtained with the *N*-methylated glycines as cosolutes. *N*-Methylglycine, *N,N*-dimethylglycine and *N,N,N*-trimethylglycine were added in a 1 molal concentration to the reaction medium. In Figure 5.4, $\ln(k_{m=1}/k_{m=0})$ is plotted versus the number of CH-groups in the nitrogen substituents. Up to n(CH)=6 additivity of the CH-group contribution is observed and the values perfectly overlap with those displayed in Figure 5.3 for Ala and Pro. This indicates that the site of substitution of the methyl group, that is to say whether it is positioned on the α-carbon atom or on the nitrogen atom, does not influence the apparent hydrophobicity of the substituent.

Table 5.3 *Natural logarithm of relative rate effects for NPTH caused by N-methylated glycines.*

Solute	**$\ln(k_{m=1}/k_{m=0})$**
N-methylglycine	-0.069
N,N-dimethylglycine	-0.139

N,N,N-trimethylglycine	-0.055

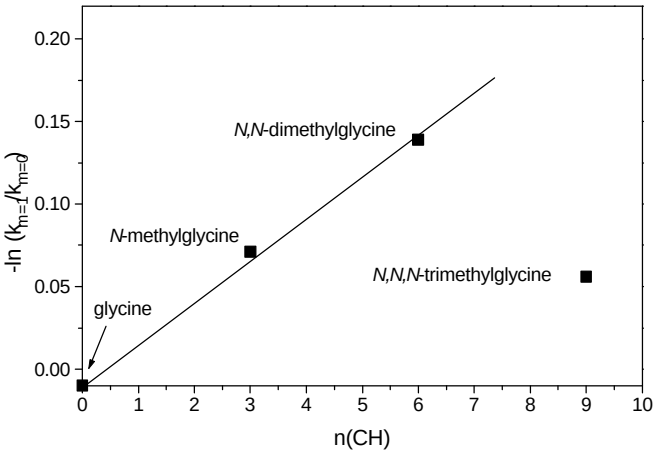


Figure 5.4 *Relationship between the number of CH-groups (n(CH)) in N-methylated glycines and their effect on the rate of hydrolysis of NPTH at 40°C and pH 11.*

A similar independence of the kinetics on the site of substitution of the methyl group was observed in Chapter 4. From a ^{17}O-NMR study of *N*-methylglycine at pH 12.5 and 40°C it appears that the line width for the carboxylate group is the same as for its α-C substituted analogue Ala[39], which suggests that the carboxylate hydration is not influenced by the presence of an α-C methyl group. This is in itself interesting because it shows again that carboxylate hydration dominates over hydrophobic hydration. On the other hand, the partial molar volume for *N*-methylglycine is greater than for Ala[16]. The contribution of electrostriction of the solvent to the partial molar volume is more negative for Ala. Thus, while the kinetics suggest that the overall hydrophobicities of the two solutes are the same, the partial molar volumes indicate that water in their hydration shells have different properties.

N,N,N-Trimethylglycine shows a significant deviation from the observed additivity in this series. Two major structural features distinguish this cosolute from the less substituted glycines. Firstly, the hydrogen bond acceptor ability of the amino group has vanished, because the non-bonded free electron pair is used to accommodate the third methyl group. Hydrogen bonding to the solvent will be diminished considerably and consequently overlap between the hydration shells of the charged/polar termini will be less. The hydration shell of the carboxylate might therefore be more developed, whereby favourable interactions with the activated complex become energetically more likely. Secondly, the solute is now a zwitterionic species. The charged ammonium group screens the methyl groups more than does the neutral amino group and therefore hydrophobic interactions of the methyl groups with NPTH are reduced in comparison with those of *N*-methyl glycine and *N,N*-dimethylglycine. These results indicate again that the hydration water of an ionic group has a more destructive influence on hydrophobic interactions than has the hydration water of non-ionic polar groups.

5.2.4 Dilute aqueous peptide solutions

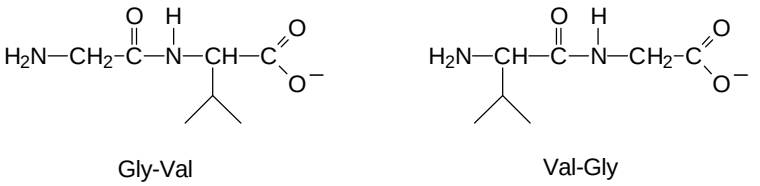
Finally, kinetic data for two dipeptides Gly-Val and Val-Gly and three glycine oligomers (diglycine, triglycine and tetraglycine) will be discussed. The results are presented in Table 5.4.

Table 5.4 *Natural logarithm of relative rate effects for NPTH caused by peptides.*

Peptide	$\ln(k_{m=1}/k_{m=0})$
Gly-Val	-0.300
Val-Gly	-0.361
Gly-Gly	+0.102
Gly-Gly-Gly	-0.188
Gly-Gly-Gly-Gly	-0.185

Since Gly does not exert a substantial effect on the hydrolysis reaction, it can be anticipated that the two dipeptide isomers will affect the rate of hydrolysis to a similar extent as Val. However, both isomers retard the reaction to a significantly larger extent as compared with Val. It is difficult to ascribe this increase in retardation to a particular structural feature in the dipeptide. It is anticipated that the peptide CONH-group contributes towards part of this retardation. In previous kinetic medium effect studies it has been shown that this group has a negative Gibbs energy group interaction parameter[41]. However, those results refer to the hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole and extending them to this study must be done with great care, because there might be probe-specific effects or effects depending on the reaction mechanism. Furthermore, the hydration shell of the amide functionality, which has now been introduced in the molecule, will interfere with those of the amino and carboxylate termini and of the Val side chain. And last but not least, the amino and carboxylate hydration spheres will show less overlap than those in the anionic amino acids because they are separated to a greater distance. In other words, there is not much certainty in how far group hydration shells are interacting with each other in these dipeptides and what the consequences for the interactions with the reactant and the activated complex are. However, the difference between the two isomeric dipeptides can be satisfactorily explained in terms of intramolecular hydration shell overlap. In Gly-Val the *iso*-propyl group experiences more shielding by the carboxylate group, *i.e* is less available for hydrophobic interactions, than in Val-Gly, where the *iso*-propyl group is more remote

from the extensively hydrated negative charge and more available for hydrophobic interactions (Scheme 5.3). The higher apparent hydrophobicity of Val-Gly results in a stronger rate retardation. The difference in hydration of Gly-Val and Val-Gly is also reflected by their apparent molal volumes, which are 122.3 and 126.5 cm^3mol^{-1}, respectively[42].This is due to differences in the contribution of electrostriction to the volume, *i.e* the contraction of solvent molecules around ammonium and carboxylate groups is different and is influenced by the presence of an alkyl substituent in the two dipeptides. A similar behaviour is exhibited by the partial molal volumes[42] and heat capacities[43] of Gly-Leu and Leu-Gly. And even with less hydrophobic alkyl substituents, like in Gly-Ala and Ala-Gly, different contributions of the methyl substituents to the overall hydrophobicity of the cosolute have been observed[44]. This was also explained in terms of the influence of the different hydration properties of the polar groups of the dipeptide on the methyl hydrophobicity.



Scheme 5.3 *Dipeptides Gly-Val and Val-Gly at pH 11.*

Once more, these results indicate that intramolecular hydration shell overlap markedly influences the noncovalent interactions of the α-amino acid side chain with the reactant.

The effects of the glycine oligomers exhibit a complex interaction pattern. Diglycine significantly accelerates the reaction whereas tri- and tetraglycine show a large and similar retardation of the hydrolysis reaction. Intramolecular hydrogen bonding interactions in short peptides in aqueous solution are highly unlikely, particularly in the absence of residues with an apolar side chain as is the case in the glycine oligomers. However, intermolecular interactions or aggregation induced by amide-amide and side chain-side chain interactions in short peptides may play a role in aqueous solution[45]. In glycine oligomers side chain-side chain interactions are absent but amide-amide intermolecular interactions in triglycine (two peptide bonds) and tetraglycine (three peptide bonds) should be considered. A small tri- or tetraglycine peptidic aggregate, or locally high concentrations of these solutes, might create a favourable hydrophobic environment for the substrate where the initial state is preferentially solvated by amide groups and is more stabilised than the transition state. If aggregation is the explanation, then the observed retardation must be an initial state effect (*i.e.* not a destabilisation of the transition state), because the reactant only ‘binds’ to the aggregate when it is favourable in terms of Gibbs energy.

However, this leaves the acceleration observed for diglycine still to be explained. From partial molar volume[15] and compressibility[46] data it can be deduced that there is a substantial increase in the electrostriction of the solvent in diglycine aqueous solutions as compared to glycine, because of the fact that the amino and carboxylate groups are separated by a longer distance. This is, of course, also true for the higher oligomers, but the effect would largely disappear when the solute molecules aggregate (by intermolecular charge-charge interactions). Aggregation in diglycine is less likely because:

1) the diglycine polar termini still interact via their hydration shells whereas in higher oligomers they are independently hydrated as was deduced from (temperature dependent) partial molar adiabatic compressibility data[18,47] and
2) the charged/polar termini in diglycine may interact with the amide group via hydrogen bonding[14]. Therefore, intermolecular amide-amide hydrogen bonding (and consequently aggregation) is less likely to occur, because the terminal groups need to be partly dehydrated, which will be enthalpically highly unfavourable.

Though these results are encouraging and give insights into the hydration of individual functional groups, it is clear that more data on simple primary and secondary amides and peptides as cosolutes are required in addition to the presented preliminary data to obtain a more complete picture of the dominant noncovalent interactions involving peptides in aqueous solutions.

5.3 Results in retrospect; comparison with literature data

It is obvious from the results presented in this chapter that the interactions of α-amino acids with 2-(4-nitrophenoxy)tetrahydropyran take place via a different molecular recognition process than with 1-benzoyl-3-phenyl-1,2,4-triazole (Chapter 4). This is not surprising taken the fact that the molecular structures of both cosolute and reactant are different in these studies. In addition the hydrolysis reactions proceed via different mechanisms. Nevertheless, the understanding of the hydration characteristics of α-amino acids has improved by the experiments described in this chapter, as long as it is presumed that the intermolecular interactions take place via the hydration shells of the interacting solutes.

Whereas in Chapter 4 pairwise interactions involving α-amino acids were dominated by the carboxylate hydration to such an extent that the hydrophobicity of apolar side chains were usually completely overshadowed, it is shown in this chapter that the ammonium hydration had its impact too; when the ammonium group

is neutralised to an amino group, the carboxylate hydration is not the only determinant in the pairwise interactions, and side chain effects are observed as well. These side chain effects show limited additivity in terms of the SWAG-approach[6], due to hydration cosphere overlapping of the different functional groups. Additivity of the pairwise interactions of the apolar CH-moieties is evident up to n(CH) = 6. This group interaction shows a reduced additivity compared to, for example, the CH_2-group contribution of alcohols and is caused by the hydration of the polar/ionic termini of the cosolutes which exert their destructive effect on hydrophobic interactions particularly in the first 1-1½ layers of hydration water. It is worth noting that, considering the fact that additivity is observed in this region, the polar/ionic hydration shells must be rather uniform.

Above n(CH) = 6 there is strong evidence for additivity of these groups. This additivity is not or at least far less influenced by the polar/ionic hydration and its pairwise interaction energy is comparable to that of the CH-groups in primary alcohols.

Whereas the SWAG-approach predicts the same group interaction parameters for structural isomers, substantial differences were observed for α-amino acids with isomeric apolar side chains. These differences can be satisfactorily explained by the dependence of branching of the chain on the distance to α-C, *i.e.* whether branching takes place inside or outside the polar/ionic hydration shells. A similar non-additive behaviour of the apolar group contributions to the kinetic medium effects for these isomers was observed in the previous chapter, though less pronounced. Without the presence of a polar group, group additivity may be observed for these isomers. However, this cannot be verified experimentally, since an apolar moiety requires a polar group to ensure sufficient solubility in water and therefore will always experience some influence by this polar group.

The results obtained for glycine derivatives and peptides are encouraging in the sense that they have shed some light on the extent of hydration of the individual polar groups and confirmed the postulate of intramolecular hydration shell interactions.

As highlighted in Chapter 1, extensive information about physicochemical solution properties of α-amino acids is available in the literature as well as numerous hydrophobicity scales, based on various solution properties. All reflect, to a certain extent, the hydration characteristics of α-amino acids. It is of interest to correlate the data presented in this chapter with some of these properties and scales to confirm that the hydration characteristics of α-amino acids as they are revealed by the noncovalent intermolecular interactions with NPTH are consistent with those recorded in literature.

When the correlations include data for Phe and β-phenylSer, estimated values for $\ln(k_{m=1}/k_{m=0})$ are used based on an linear extrapolation to 1 molal concentration, since the solubility of these solutes did not allow measurements at this concentration. It has to be kept in mind that these values are the lower limits of the expected retardations as the concentration dependence of ln k is not linear.

Correlations with partial molar properties

As was pointed out in Chapter 1, volumetric properties of solutes in aqueous solution, like the partial molar volume, compressibility and expansibility are useful thermodynamic properties because they are sensitive to the degree and nature of solute hydration.

The partial molar volumes correlate reasonably well with the kinetic solvent effects (Figure 5.5*a*), though the differences between isomers are not clearly reflected by the volumes. There are two reasons for this disagreement:

1) The partial molar volume is a composite of the intrinsic volume and a volume due to solvent-solute interactions, where the intrinsic part is by far the largest, but probably more important is
2) the fact that these are volumes for the zwitterionic species, and effects of alkyl groups on the volume of the ionic ammonium group are considerable and work opposite to the effects of alkyl groups on the volume of the carboxylate group[48].

The latter is illustrated in Table 5.5 where partial molar volumes of isomeric butylcarboxylic acids, butylcarboxylate ions and butylammonium ions are displayed. As can be seen, the effects of alkyl chain branching on the volume of carboxylic acids is non-existent, whereas there is a decrease in volume with branching nearer to the ionic group for the carboxylate ions and an increase for the ammonium ions. This is due to differences in electrostriction around the two charged groups.

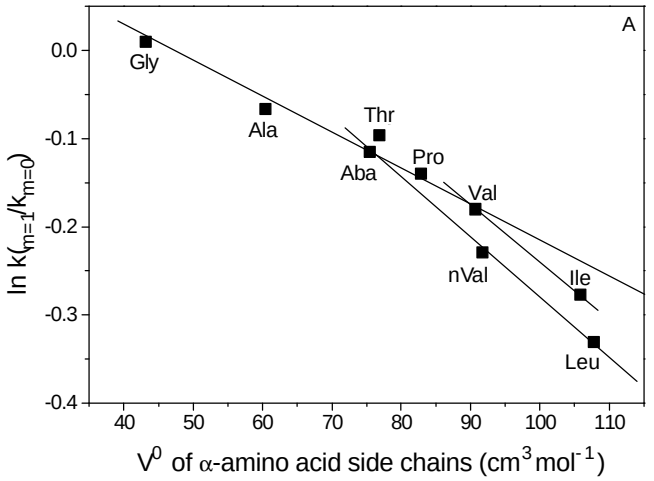
Table 5.5 *Partial molar volumes of isomeric butylcarboxylic acids[a], butylcarboxylate ions[b] and butylammonium ions[b] ($cm^3\ mol^{-1}$) in water at 25°C.*

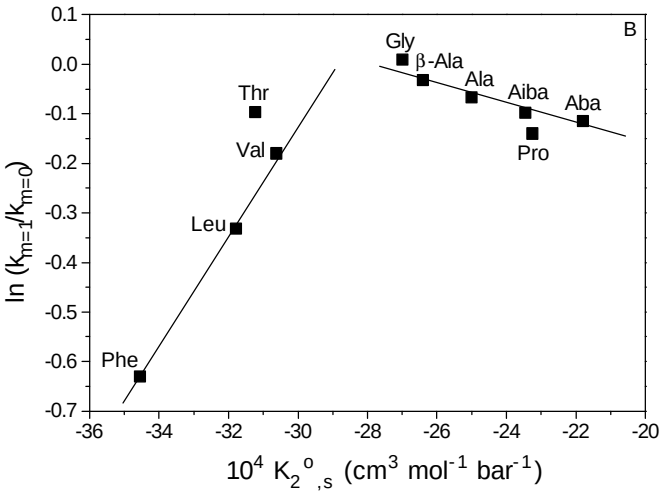
R	RCO_2H	RCO_2^-	RNH_3^+
n-butyl	100.5	92.3	80.1
iso-butyl	100.5	92.0	80.1
sec-butyl	100.5	90.0	80.8
tert-butyl	100.5	88.9	82.2

[a]ref. 49, [b]ref. 50

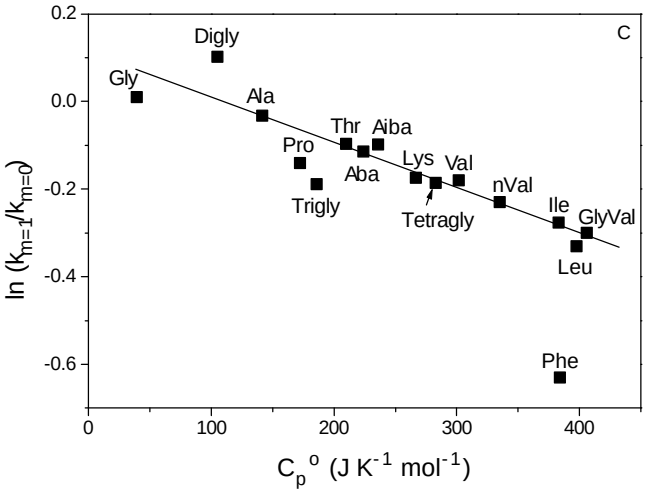
Electrostriction around NH_3^+ is large, around CO_2^- small[1,48,51] and therefore reduced electrostriction dominates the volume change for the ammonium ions and reduced

hydrophobic hydration dominates the volume change for the carboxylate ions. In α-amino acid zwitterions these volume effects probably cancel, whereas in the anionic α-amino acids the electrostrictive effect of the ammonium group is absent. This accounts for the lack of correlation with the kinetic data. Correlation with partial molar isentropic compressibilities is worthwhile, because the intrinsic volume can be regarded as incompressible, and changes in compressibility will therefore reflect solvent-solute interactions. As can be seen in Figure 5.5*b,* the relation is peculiar in the sense that it appears that the α-amino acids can be divided into two groups;





one going from Gly → Aba where an increase in apolar chain length causes an increase in compressibility and one from Gly → Phe where an increase in hydrophobicity of the side chain causes a decrease in compressibility. Though not all compressibility data on α-amino acids agree extremely well, this feature is also visible from other compressibility data[22]. Water around apolar groups is less



compressible than pure water($K°_s(H_2O)$ = +8.17 x0 10^4 cm^3 mol^{-1} bar^{-1}) and the trend for Gly

Figure 5.5 *Correlations between observed rate retardations and partial molar (A) volumes (B) isentropic compressibilities and (C) heat capacities for amino acids. V and C_p data are retrieved from reference 52, K data from reference 46.*

→Phe is therefore anticipated. However, water around ionic groups is even less compressible. It is speculated that the hydrophobic hydration shells in the series Gly to Aba are not developed (or too badly to be detected by compressibilities measurements) and do not show up. The trend then reflects a decrease in extent of ionic hydration layers, due to intramolecular hydration shell overlap. In addition it has to be kept in mind that the correlation is between anionic and zwitterionic species and as for the volumes, compressibilities reflect electrostrictive effects, which are different for the anionic and zwitterionic α-amino acids. The partial molar heat capacity (the second derivative of the Gibbs energy towards temperature) is an important thermodynamic parameter because hydrophobic hydration water has a very large heat capacity and correlation with this parameter may reflect the hydrophobic nature of the solute. The correlation is shown in Figure 5.5*c*, and is even better than the correlations with partial molar volumes and compressibilities. It supports the notion that hydrophobic interactions govern the molecular recognition process determining the kinetic solvent effects.

Correlations with some hydrophobicity scales for α-amino acids

The choice of hydrophobicity scales with which the kinetic data have been correlated has been quite random, but it was aimed to chose scales in such a way as to present divergent methods and techniques. The most referred to α-amino acid hydrophobicity scale is the one provided by Nozaki and Tanford[53] which is based on

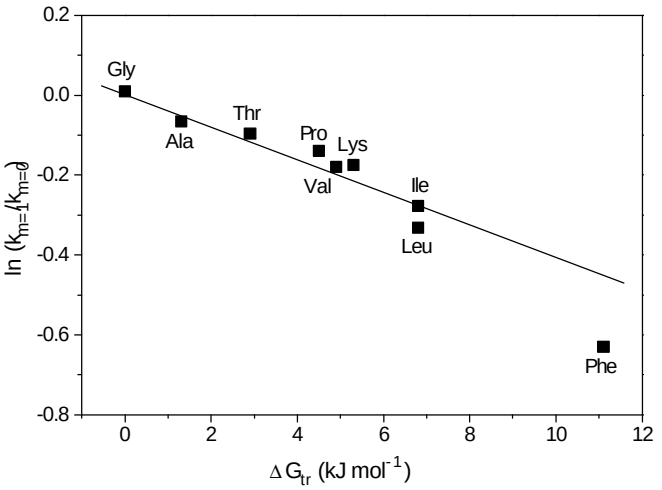


Figure 5.6 *Correlation between the kinetic medium effects for NPTH and Gibbs energies of transfer of amino acid side chains from ethanol to water at 25°C (data retrieved from ref. 53).*

Gibbs energies of transfer of amino acid side chain from 100% organic solvent to water. Though data for only a limited number of α-amino acids have been reported, it was felt that a correlation with this scale could not be left behind. With the exception of Phe, the correlation is linear (Figure 5.6) and once more this indicates that the hydrophobicity of the α-amino acids is well reflected by the kinetic medium effects. Other hydrophobicity scales which are based on solubility data (like the one by Nozaki and Tanford[53]) are those reporting the 1-octanol/ water partition coefficients, P-values, of free zwitterionic α-amino acids[54] or α- amino acid side chains in tetrapeptides[55]. The log P-values allow the definition of a hydrophobicity (or lipophilicity) constant π for the α-amino acid side chain. Correlations with these values are slightly (and similarly) curved for both studies. Again the data for Phe and also Lys do not correlate well.

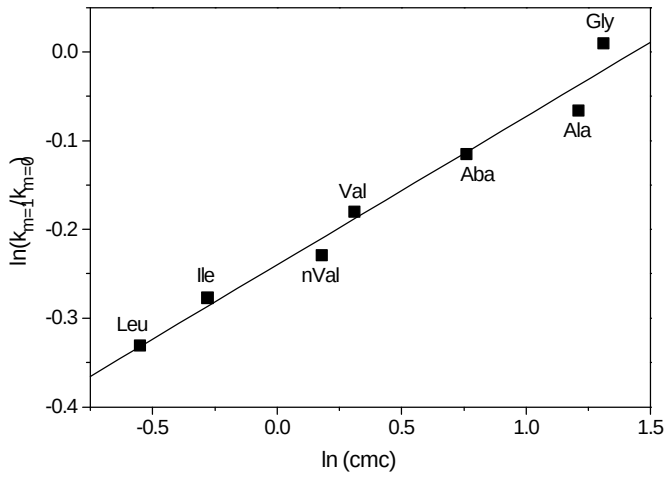


Figure 5.7 *Correlation between kinetic medium effects for NPTH and ln(cmc) of N-dodecanoyl amino acids (data from ref.35).*

Miyagishi *et al.*[35] based their hydrophobicity scale on the cmc values for the sodium salts of *N*-dodecanoyl -amino acids. Since the Gibbs energy (and therefore hydrophobicity) of micellisation is proportional to ln(cmc), the kinetic results were plotted versus this value. The correlation is remarkably linear and even the differences between the structural isomers (Val/nVal and Leu/Ile) show up to the same extent (Figure 5.7). The same holds for a scale based on R_f-values ofα-amino acids on silicagel by Thin

Layer Chromatography with an alkaline aqueous mobile phase[56] where the more hydrophobic α-amino acids have higher R_f-values; a good correlation is observed (except for Phe and Lys) including the structural isomers (not shown in a figure).

There are several hydrophobicity scales which are based on properties of amino acid side chains in proteins. They are obtained, for example, by studying the stability of a protein by site-directed mutagenesis or by determining the proportion of residues which are 95% buried into the interior of a series of proteins[57] or the ratio of buried to accessible molar fractions of the residues[58]. Correlations with the latter two are bad. This can be explained by the fact that the conformation and hydration of a protein (and indirectly whether a residue is buried or not) is not solely determined by hydrophobic interactions. It is therefore important to realise that information on the hydration of amino acids is only a first step towards the understanding of the conformation and stability of a protein.

Correlation with dynamic hydration numbers

Hydration numbers for α-amino acids can be obtained by several methods. The absolute values are dependent on the experimental technique, because they depend on the definition of the hydration number, but trends are useful in correlations. Here, a correlation is made with dynamic hydration numbers as obtained from ^{17}O-NMR T_1 relaxation times for aqueous α-amino acid solutions[30]. The hydration number is in this case determined by the thermal motion of water molecules which is restricted around hydrophobic and more rapid around hydrophilic groups. A correlation with these hydration numbers appears to exist (Figure 5.8), suggesting that the hydration properties of α-amino acids are well reflected by the

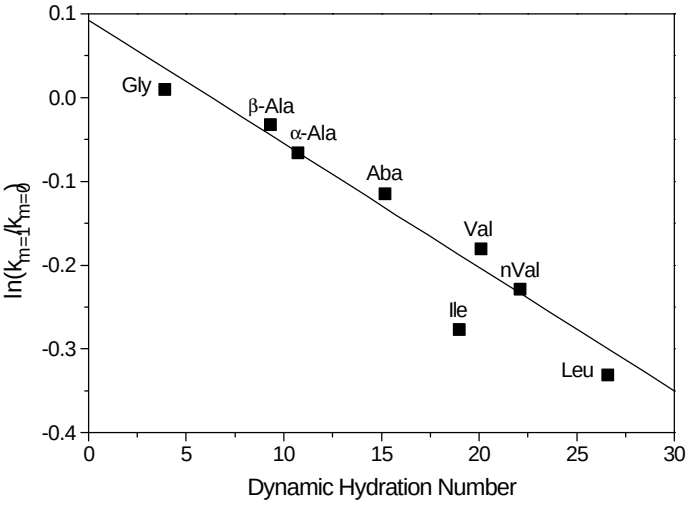


Figure 5.8 *Correlation of the kinetic medium effects with the dynamic hydration number as obtained from ^{17}O-NMR T_1 measurements (data retrieved from ref. 30).*

kinetic experiments.

Correlation with intrinsic properties of the solute and macroscopic properties of the solvent

Now that correlations of the kinetic data with solution properties of α-amino acids are shown to exist and are generally satisfactory, which qualifies the kinetic method as a valuable tool for the qualification and quantification of hydrophobic interactions, the question remains if the classification of the α-amino acids in terms of hydrophobicity can be carried back to an intrinsic property of the solute.

Charton has suggested and shown that many of the hydrophobicity scales are in fact a function of the polarisability of the amino acids[59]. In Figure 5.9 the medium effects are plotted versus the polarisability and it turns out that the relation is only partial. As the polarisability is the same for structural isomers, the kinetic differences observed for isomers are not reflected by the polarisability of the solute. Furthermore, Pro and Thr do not fit in. Phe and Lys are deviating significantly as was observed in most of the other correlations. The relationship between the kinetic results and dielectric increments[60], dipole moments and Kerr constants[61] (Kerr constants are obtained from optical birefringence data of aqueous amino acid solutions and depend on the dipole moment and the anisotropic polarisability tensor) were not obvious. Since the activated complex in the transition state of the hydrolysis reaction shows an increase in polarisation compared to the reactant, it may be expected that the Gibbs energy of activation is affected by the relative permittivity of the solution. Though dielectric increments are positive and large for zwitterionic amino acids, they are much smaller for the anions and cations[62] and therefore the stabilisation of the polarised transition state is probably less significant in the case of 2-(4-nitrophenoxy)-tetrahydropyran than in the case of 1-benzoyl-3-phenyl-1,2,4-triazole (previous chapter). In addition, dielectric increments and dipole moments are very similar for all α-amino acids and do not

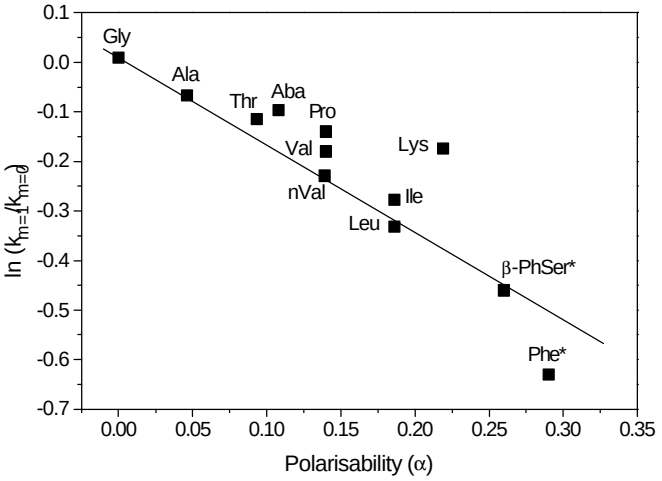


Figure 5.9 *Correlation of the kinetic medium effects with solute polarisabilities (data retrieved from ref. 59).*

explain the observed trends. Finally, it should be kept in mind that, as emphasised in the previous chapter, the relative permittivity of the solvent system is a macroscopic solvent property, which might not reflect the dielectric behaviour of the solvent in the close vicinity of the ions at all.

In many of the correlations, phenylalanine and lysine show clear deviations from the linear relationships obtained. Lysine is the only amino acid with a polar ionic side chain that has been examined. Its hydrophobicity turns out to be less than anticipated from the CH-group additivity approach, but it is not hydrophilic as it appears from many hydrophobicity scales. Presumably polar interactions of the side chain of Lys are reflected in those hydrophobicity scales while they are not in the kinetics. The apparent hydrophobicity of Phe is always higher as it is obtained from the kinetic experiments than from the hydrophobicity scales in the literature. As mentioned previously, there appears to be a different molecular recognition process between the amino acid aromatic group with NPTH compared to amino acid aliphatic groups. If it was solely due to the pronounced hydrophobicity of these solutes, correlations with hydrophobicity scales would be better than is observed here and this leads to the conclusion that other noncovalent intermolecular interactions must play a role in addition to hydrophobic interactions.

In conclusion, it can be stated that the hydration properties of α-amino acids (*i.e*. the solute-solvent interactions) as they appear from the kinetic solvent effects agree well with literature data which are presumed to reflect α-amino acid-water interactions. The hydrolysis of the acetal appears to be a valuable (indirect) way through which these interactions can be measured. It appears that there are only a few cases where noncovalent α-amino acid-probe interactions other than hydrophobic interactions govern the medium effect and disturb the observed trends. With this knowledge an extension of this study to studies of amides and peptides as cosolutes is deemed worthwhile.

5.4 Experimental procedures

Materials. All amino acids and peptides were purchased from Janssen Chimica, Fluka and Sigma and were used without further purification (purity of 99% or higher). 2-(4-Nitrophenoxy)tetrahydropyran (NPTH) was synthesised according to a literature procedure[2].

Kinetic measurements. Kinetic measurements were carried out following the procedures described in Section 2.7, however at 40.0°C ± 0.05°C. Aqueous solutions were adjusted to pH 11 with a sodium hydroxide solution and were prepared immediately prior to use. All measurements were performed with blank reactions (*i.e*. water reactions) in the same kinetic run. Excellent first-order kinetics were obtained by following the change in absorbance at 400 nm. Rate constants were reproducible to within 1% for solutions containing the α-amino acids and 2% for the peptides and alcohols.

5.5 Acknowledgement

Silvia Portolan is acknowledged for the kinetic measurements of the alcohols which she performed within the framework of the Erasmus exchange program.

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Appendix

A.1 A preliminary study to further investigate α-amino acid hydration

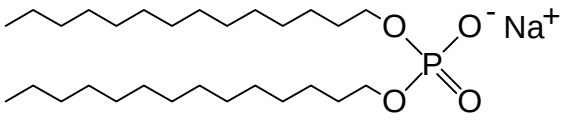
In a preliminary study, the effects of zwitterionic α-amino acids (and some peptides) on vesicle bilayer fluidity have been measured to further investigate the hydration properties of these solutes by a method other than kinetic solvent effects. Fluorescence depolarisation spectroscopy has been employed to study these effects and results are discussed in terms of noncovalent interactions between α-amino acids and the vesicle bilayers.

A.2 Effects of zwitterionic α-amino acids and of peptides on vesicle bilayer stability

In the previous chapters, *pairwise* interactions involving α-amino acids in aqueous solution have been described. Now, attention is turned to interactions of α-amino acids (and some peptides) with hydrophobic assemblies in aqueous solution. The formation of molecular assemblies in water from amphiphilic molecules, such as closed bilayers (vesicles) is driven by noncovalent interactions (bulk hydrophobic interactions) between the hydrocarbon chains and is accompanied by an increase in entropy[1]. The aggregate morphology depends on the cross-sectional surface area of the headgroup and on chain length and volume[2]. In these aggregates, the polar headgroups of the amphiphiles have favourable interactions with the solvent water and ensure solubility. The hydrocarbon chains, however, cannot have favourable interactions with the water molecules and are shielded from contact with water in the core of the aggregate.

When the amphiphilic assembly consists of phospholipids, it models a biomembrane. A real biomembrane, however, is more complex. It contains, for example, proteins and peptides, glycolipids and cholesterol. The noncovalent interactions and modes of attachments of the proteins and peptides with the vesicle bilayer depend on their α-amino acid sequence[3]. Hydrophobic interactions take place between the peptide (or protein) side chain and the hydrocarbon chains of the lipids and are, for example, responsible for the permeability of the membrane by forming trans-membrane ion-channels[4]. The perturbation of lipid bilayers due to protein-lipid interactions has been investigated in some detail[5]. Also, the relationship between hydrophobicity of naturally-occurring and synthetic peptides

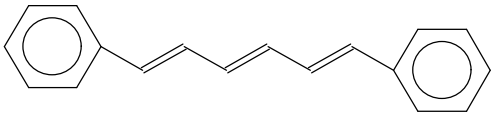
and their interactions with (model) biomembranes has been studied[3]. However, the effects of interactions of small molecules, like α-amino acids and small peptides (number of residues < 5), on the properties of (model) biomembranes have been scarcely explored[6], even though in tissues and cytoplasm of all cells there is a continuous presence of free α-amino acids, the so-called "amino acid pool". Furthermore, α-amino acids are transported across the cell membrane[7]. In other words, it is relevant to study the interactions of biomembranes with α-amino acids. The studies that have been undertaken[6] suggest little perturbing effects of α-amino acids on bilayer stability. However, it was felt that this subject has not been studied in sufficient depth and therefore we took the initiative to study these effects more extensively. As a simplified model membrane, vesicles formed from synthetic amphiphiles have been employed. Vesicles formed from synthetic amphiphiles can successfully mimic structural and functional aspects of biological membranes[8]. The sodium salt of di-*n*-



NaDTP

tetradecyl phosphate (NaDTP) was chosen for this purpose, since the properties of vesicles formed from this amphiphile are well investigated[9] and are suitable for the proposed study (see Section A3).

One of the characteristic properties of vesicle bilayers is that they show a main phase transition temperature at which the bilayer changes from a rigid gel-like structure (L_β), with all-trans rotamers in the alkyl chains, to a fluid liquid-crystalline structure (L_α), containing also gauche rotamers in the alkyl chains. The temperature at which this transition occurs depends crucially on the molecular geometry of the amphiphile[10]. The transition is accompanied by a heat effect and can therefore be examined by differential scanning microcalorimetry (DSC). Other methods to determine $T_{l\rightarrow g}$ include the investigation of the head group mobility (in the case of di-*n*-alkylphosphates by ^{31}P-NMR) or the change in bilayer packing, using fluorescence depolarisation techniques. For the latter, *all-trans*-1,6-diphenyl-1,3,5-hexatriene (DPH) is often used as a hydrophobic fluorescent probe molecule which can be incorporated into the hydrocarbon core of the vesicle bilayer[11]. DPH is excited at a specific wavelength. The fluorescence of the DPH molecules in the liquid-crystalline phase will be depolarised, because the molecules have a high mobility. In the gel-like state, however, the molecules have much less freedom to move and the fluorescence will only be slightly depolarised[12].



DPH

The interactions of added solutes with the vesicles (hydrophobic interactions with the bilayer interior as well as electrostatic interactions with the negatively charged phosphate headgroups) can perturb the bilayer packing and consequently affect the $T_{l\rightarrow g}$ or bilayer fluidity. In the preliminary study presented here, the main aim was to study changes in the gel to liquid-crystalline phase transition temperature of NaDTP vesicles due to interactions with α-amino acids and some dipeptides employing the DPH fluorescence depolarisation technique. In addition a few experiments using DSC have been performed.

NaDTP was also chosen for its relatively high $T_{l\rightarrow g}$ (54°C), which is convenient because a reduction of $T_{l\rightarrow g}$ is anticipated in the presence of the additives. Secondly, the phase transition for NaDTP is very cooperative and therefore small changes in $T_{l\rightarrow g}$ can still be observed accurately.

$T_{l\rightarrow g}$ values have been measured in the presence of 0.15 molar concentrations of several α-amino acids and dipeptides at pH 7.4, *i.e*. where the solutes are in their zwitterionic form.

The results obtained by fluorescence depolarisation measurements are shown in Table A.1. The change in polarisation as a function of the temperature is shown for several additives in Figures A.1 and A.2. As can be observed from the

Table A.1 *Effects of several α-amino acids and short peptides on the phase transition temperature of vesicles bilayers formed from NaDTP at pH 7.4 (buffered).*

Solute added (0.15 M)	$T_{g\rightarrow l}$ (°C)[a]	P_{max}[b]	Cooperativity[c]
None	54	0.394	1
Glycine	56	0.380	0.8
Valine	56	0.375	0.7
Serine	57.5	0.391	1.1
Leucine	53.5	0.351	1.1
Phenylalanine	52	0.344	1.1
Phenylalanine amide	~15-35	0.310	0.5
Diglycine	57	0.382	0.9
Triglycine	51	0.372	1.7
Val-Gly	46.5	0.187	0.3
Gly-Val	-	0.12	-

[a]Point of transition with highest slope. [b]Taken at a temperature where P has levelled off. [c]Relative to control (i.e. where no α-amino acid was added). Cooperativity was determined by measuring the depth of the minimum of the first derivative of the curves with respect to temperature.

first column in Table A.1, $T_{l\rightarrow g}$ is not significantly affected by the presence of the short-chain α-amino acids, which indicates their hydrophilic character, a feature which was also observed in Chapter 4. Since the bilayers are negatively charged, a strong interaction between the ammonium groups of the α-amino acids and the phosphate head groups is anticipated. It is assumed that in the case of Gly and Ser (which can be viewed as relatively hydrophilic species[13]), only interactions with the headgroups take place. Even the possibility of the Val side chain to interact with the NaDTP alkyl chains appears to be hampered due to the extensive hydration of ammonium and carboxylate groups. The slight increases in $T_{l\rightarrow g}$ of these additives can possibly be explained in terms of expulsion of Na^+ counterions from the headgroup region and dehydration of the phosphate headgroups (the ionic groups of the α-amino acids and the phosphate will compete for water). The cross-sectional surface areas of the hydrated headgroups reduce and bring the phosphate groups in closer contact. This is particularly clear in the case of Ser which can hydrogen bond to the phosphate head groups. Presumably, the dehydration of the head groups causes a closer packing of the alkyl chains as well, leading to increased bilayer stability. Previously it was shown that dehydration of vesicle bilayers of di-*n*-alkyl phosphates can induce a more efficient bilayer packing[14]. The effects, however, are modest, indicating only little distortion of the hydrophobic bilayer interior. The effects on the phosphate head groups may be more pronounced and could be investigated further with ^{31}P-NMR.

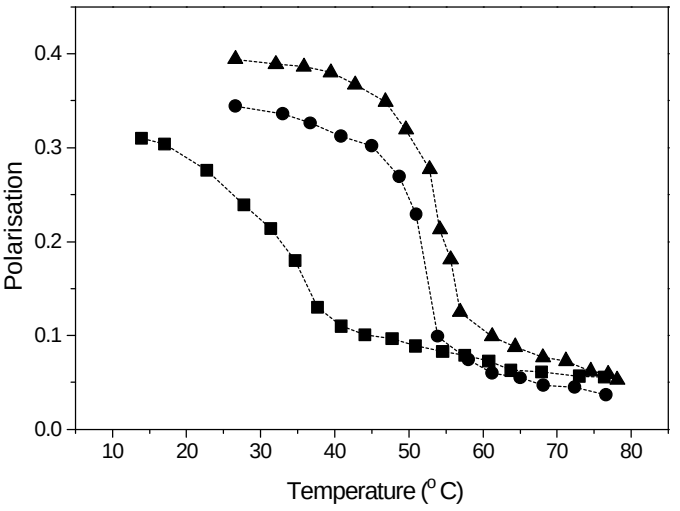


Figure A.1 *Fluorescence depolarisation results for NaDTP vesicles in water (▲), Phe (●) and Phe-amide (■) aqueous solutions at pH 7.4.*

On the contrary, the more hydrophobic α-amino acids Leu and Phe cause a decrease in $T_{l\rightarrow g}$, which indicates a reduction in bilayer stability. This can be satisfactorily explained in terms of hydrophobic interactions with the apolar NaDTP side chains. The Leu and Phe side chains are large and sufficiently hydrophobic to interact with hydrophobic binding sites at the surface of the NaDTP vesicle bilayers.

These interactions are sufficiently strong to induce distortion of the bilayer packing and therefore a reduction in phase transition temperature.

The result obtained for phenylalaninamide as an additive, which shows a very low (and noncooperative) transition, suggests that the effects of Leu and Phe are counteracted by the negatively charged carboxylate group, which has repulsive electrostatic interactions with the negatively charged phosphate group. In the case of phenylalaninamide, this negative charge is absent and therefore the hydrophobic part of the molecule has better opportunities to interact with hydrophobic binding sites at the bilayer surface, on one hand due to less repulsion between the additive and the bilayer surface, on the other due to less intramolecular hydration shell overlap within the additive[15].

The intercalation of the hydrophobic parts of the latter three additives into the bilayer is confirmed by the maximum polarisation values achieved. These are significantly lower than for the first 4 entries of Table A.1. The hydrocarbon chains of NaDTP have a relatively high mobility even below $T_{l\rightarrow g}$ for the Leu, Phe and phenylalaninamide aqueous solutions. This can only be explained by inefficient packing of the alkyl chains due to hydrophobic interactions with these solutes. The question remains, however, whether these interactions are only operative in the gel state or in both gel and liquid-crystalline states. It is quite plausible to assume that it affects only the gel state, since in the liquid-crystalline state the hydrocarbon chains are much 'floppier' and less densely packed.

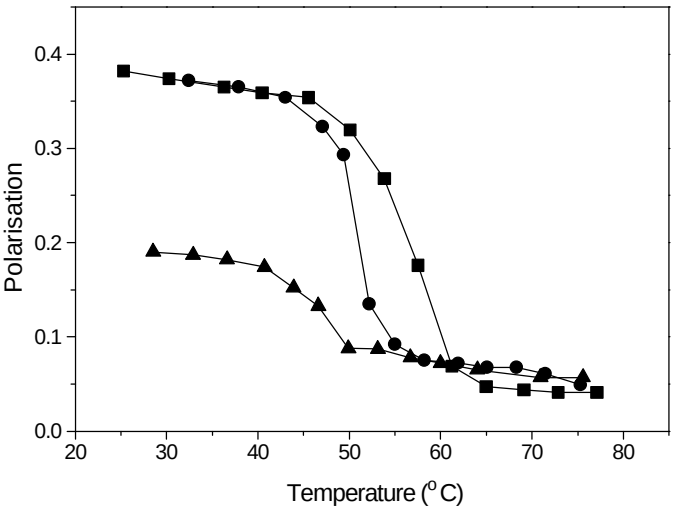


Figure A.2 *Fluorescence depolarisation results for NaDTP vesicles in diglycine (■), triglycine (●) and Val-Gly (▲) aqueous solutions at pH 7.4.*

The effects of di- and triglycine on $T_{l\rightarrow g}$ have also been determined. The $T_{l\rightarrow g}$ values decrease from diglycine→glycine→triglycine, where diglycine and glycine slightly stabilise, whereas triglycine significantly destabilises the vesicle bilayer.

In Chapter 5 a similar trend in the kinetics of the unimolecular hydrolysis of 2-(4-nitrophenoxy)tetrahydropyran was observed, where Gly had a negligible effect on the reaction rate, whereas diglycine accelerated and triglycine decelerated the

reaction rate. Obviously, diglycine is very hydrophilic; the amide bond and the two CH_2-moieties are not sufficiently hydrophobic to undergo hydrophobic interactions with apolar sites at the bilayer surface. By contrast, triglycine is able to bring about a significant reduction in bilayer stability. However, the maximum polarisation value is not significantly reduced with respect to the neat vesicle bilayers. Probably only the phosphate headgroups are involved in the intermolecular interactions, which cause a reduction in $T_{l\rightarrow g}$ without affecting the bilayer packing considerably. Therefore, it is believed that the interactions of triglycine with the DTP vesicle bilayers are not of hydrophobic origin either. The different behaviour of diglycine and triglycine must be attributed to amide-phosphate interactions in the case of triglycine, which do not take place in the case of diglycine. This assumption is supported by the fact that in diglycine, the carboxylate and ammonium hydration shells still overlap[16], *i.*e. the hydration of the intermediate amide bond is poorly developed and its interactions with the phosphate groups are diminished. Finally, the values of $T_{l\rightarrow g}$ for Val-Gly and Gly-Val aqueous solutions in aqueous solutions have been measured. They show a remarkable difference with respect to the glycine dipeptide. Val-Gly causes a reduction in $T_{l\rightarrow g}$ of almost 8°C. Clearly, the *iso*-propyl side chain has a more destructive effect on the bilayer in Val-Gly than in Val. This is due to the neighbouring carboxylate group in Val, whose hydration shell reduces the possibilities of the side chain to interact with the vesicle bilayer via hydrophobic interactions, a phenomenon repeatedly observed in the previous two chapters. In Val-Gly, the carboxylate group is three atoms more remote from the side chain than in Val and therefore repulsion with the negatively charged phosphate groups is reduced. Again P_{max} is reduced considerably (to only half the value of P_{max} for the neat vesicles), indicating that the interactions of the iso-propyl group affect the bulk hydrophobic interactions between the DTP alkyl chains.

The result with Gly-Val is more difficult to explain. The *iso*-propyl group is now quite remote from the ammonium group, *i.e*. far from the part of the solute which interacts with the bilayer surface. An effect smaller than that for Val-Gly was anticipated. However, no $T_{l\rightarrow g}$ was observed at all and the polarisation value increased gradually and linearly with decreasing temperature, reaching a P_{max} of only 0.12. This reproducible result seems to indicate a total disruption of the vesicles. At present, it is unclear why this occurs. A quick and reliable method to obtain more insight into the morphology of the vesicle bilayers in the presence of the additives and to check whether or not molecular aggregates are present at all in the case of Gly-Val is transmission electron microscopy. These experiments have not yet been carried out.

Overall, it appears that the results obtained are consistent with those presented in Chapters 4 and 5. Apparently, the interaction mechanisms of the α-amino acids and peptides with the kinetic probes and the vesicle bilayers have common features and reflect the hydration characteristics and hydrophobicity of these solutes.

Differential scanning calorimetry (DSC) is a powerful and accurate method to determine the $T_{l\rightarrow g}$. The DSC experiments which have been carried out on DTP/α-amino acid solutions did not show effects on $T_{l\rightarrow g}$ except for phenylalaninamide, of which the reduction of the $T_{l\rightarrow g}$ of the DTP vesicles was very clear. A decrease of almost 40°C was observed and the transition was noncooperative, in agreement with the polarisation measurements. Moreover, analysis of the transition in terms of patches of bilayers undergoing the transition revealed that phenylalaninamide is present in clusters in the bilayer. This self-aggregation is probably induced by the presence of the bilayer, since bulk hydrophobic interactions of phenylalaninamide are not observed in the absence of a bilayer (see the kinetics results in Chapter 4). When concentrated at the bilayer surface, phenylalaninamide molecules obviously benefit more from interactions with themselves than with the bilayer, most probably due to favourable polar amide-amide hydrogen bonding interactions. The amide groups are already partly dehydrated at the bilayer surface, stimulating the formation of intermolecular C=O•••H-N hydrogen bonds. It is anticipated that a similar molecular recognition process takes place in the case of diglycine, Val-Gly and Gly-Val peptides, but these additives have not been investigated with DSC.

The DSC measurements also showed that the vesicle-α-amino acid solutions have high stabilities. Repeatedly heating and cooling of the solutions showed the reversibility of the phase transition and did not change the features of the transition significantly.

This preliminary study provides additional information about the hydration of α-amino acids and short peptides. It is worthwhile to further investigate the interactions of these solutes with (model) biomembranes. There are many versatile techniques available (fluorescence spectroscopy, electron microscopy, DSC, NMR-techniques) which can elucidate different aspects of the interaction mechanisms. In addition, studies should be extended to positively charged and nonionic vesicle systems in order to obtain more insights into the interactions of the different functional groups of the α-amino acids with the amphiphilic headgroups.

A.3 Experimental procedures

Materials. Di-*n*-tetradecylphosphate (sodium salt) was prepared as described previously[9].
All other chemicals have been purchased and are of the highest purity available (generally 99+%). They were used without further purification. All water used was doubly distilled in an all-quartz unit.

Vesicle preparation and fluorescence depolarisation measurements. Vesicles were prepared by pulsed sonication of 5 mg of di-*n*-tetradecylphosphate in a 2 ml Hepes/NaAc (5mM) buffer solution at pH 7.4 for ca. 2 minutes at 70°C (until clear solutions were obtained). The vesicle solution was diluted into the 0.15 M solution of the α-amino acid or peptide of interest (in Hepes/NaAc buffer) at 70°C to a final amphiphile concentration of 5 x 10^{-5} M. The required amount of DPH in THF was injected to yield a final DPH concentration of 5 x 10^{-8} M. The solution was allowed to stand at 70°C for about 5 minutes before use while being stirred.

No differences in results were observed between vesicles which were prepared in the neat buffer or in buffer containing the solute of interest.

The fluorescence depolarisation measurements were performed with a SLM Aminco SPF 500C spectrofluorometer equipped with a thermostated cell holder. DPH was excited at 360 nm; the emission wavelength was 428 nm. The degree of fluorescence polarisation (P) was calculated from:

$$P = \frac{I_{parallel} - I_{perpendicular}}{I_{parallel} + I_{perpendicular}}$$

where the I-values are the fluorescence intensities determined with the polarisers oriented parallel and perpendicular to the direction of polarisation of the excited radiation.

Vesicle solutions were cooled down by 3-4° steps and were allowed to equilibrate for 15-20 minutes. P values were determined in quintuple and averaged.
The $T_{g\rightarrow l}$ for the neat DTP-vesicles deviates from the literature value[17], due to a different method of vesicle preparation.

Differential scanning calorimetry. DSC experiments have been carried out according to previously described procedures[18].

A.4 Acknowledgements

Barbara Briggs is acknowledged for carrying out the differential scanning calorimetry experiments at Leicester University (U.K.); Anno Wagenaar for synthesising sodium di-*n*-tetradecylphosphate.

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CHAPTER 6

Comments and Concluding Remarks

6.1 Introduction

This thesis has dealt primarily with kinetic medium effects, used to quantify and qualify intermolecular noncovalent interactions in aqueous solution. In Chapters 2-5, kinetic medium effects involving cosolutes which possess both an apolar alkyl moiety and a polar moiety have been investigated. In Chapter 2, this polar moiety was an amide group, in Chapter 3 the cationic ammonium group, in Chapter 4 the zwitterionic group of α-amino acids and in Chapter 5 the deprotonated form of the α-amino acid. The polar groups showed different effects on the apparent hydrophobicity of the alkyl groups attached to them, due to interactions between the incompatible hydration shells of the functional groups within the solute molecule. Since it was the alkyl part in the molecule that was varied in size, the emphasis has been primarily on the quantification of the hydrophobic interactions. In this final chapter, the kinetic results of Chapters 2-5 are compared and the polar group contributions to the kinetic medium receives more attention.

Kinetic medium effects on different hydrolysis reactions highlighted the specificity of solute-solute interactions. The results are viewed in terms of these probe-specific effects.

The major part of the thesis has concentrated on α-amino acid hydration. Implications of the results with respect to protein folding and stability will be discussed briefly.

Investigation into the possibility of diastereomeric interactions involving the chiral enantiomers of α-amino acids has not been rewarding. A few words are devoted to these endeavours.

6.2 Kinetic solvent effects revisited

Probe-specific effects

In Chapters 2-5 different (water-catalysed) hydrolysis reactions have been used for the study of kinetic solvent effects. Especially the results described in Chapter 2 and a comparison of the results in Chapters 4 and 5 show that kinetic medium effects are quite dependent on the nature of the kinetic probe. This conclusion is of course

not surprising, since the probes have different hydrophobicities and provide different sites for interactions with the cosolutes in the initial and transition state. But the kinetic effects do not always show what is anticipated on the basis of hydrophobicity of the probe (and of the interacting cosolute), *i.e*. one cannot simply say that a more negative G(c) indicates increased hydrophobic interactions between kinetic probe and cosolute. There is often a subtle interplay of several types of noncovalent interactions. Although hydrophobic interactions are unique for aqueous solutions, the importance of other noncovalent interactions in aqueous media cannot be ruled out and in some cases they dominate the kinetic medium effects.

A clear example of kinetic medium effects which do not reflect the hydrophobicity of the kinetic probe was described in Chapter 2, where polar stabilising interactions in the transition state for the hydrolysis reactions are weaker for the less hydrophobic probe, giving rise to more negative G(c) values for the less hydrophobic probe.

Another example comes from the comparison of the kinetic medium effects of the isomers valine and norvaline on different probes with different hydrolytic mechanisms (Chapter 4 and 5). Valine has the lower G(c) value for the experiments reported in Chapter 4, whereas in Chapter 5 norvaline shows the larger retardation. These results appear contradictory at first sight. Actually, norvaline is the more hydrophobic α-amino acid[1], as is well reflected by the kinetic medium effects for the reaction described in Chapter 5. In Chapter 4, however, electrostatic interactions of ionic groups with the transition state are dominating and overwhelm hydrophobic interactions. The side chain of norvaline perturbs the ionic hydration shell (and subsequently the accelerating effect) less than does the side chain of valine. Also, a comparison of the effects caused by Ser and Thr and by the isomers Ile and Leu in both chapters shows that in Chapter 4 the kinetic medium effects principally reflect changes in ionic group hydration, whereas in Chapter 5 they reflect primarily the hydrophobicity of the side chain.

It is important to recognise the interplay of hydrophobic and hydrophilic intermolecular interactions. Measurements of medium effects on the reactivity of several kinetic probes improves the understanding of the significance of overlapping hydration spheres, even though at first sight it seems to complicate the interpretations.

Apolar group contributions and intramolecular hydration shell overlap effects

Difficulties are sometimes encountered when one attempts to describe the kinetic medium effects in terms of pairwise CH_2-group interactions based on the SWAG-approach[2]. In Chapters 2,3 and 5, the majority of the kinetic medium effects could

still be satisfactorily analysed in terms of additive CH_2-contributions inside and/or outside the hydration sphere of the polar group, where the extent of the pairwise interactions depends on the extent of hydration of the polar group (already violating one of the assumptions of the approach, see Chapter 1). Incompatible hydrophilic and hydrophobic hydration shells lead to a reduction in apparent hydrophobicity of the solutes. In Chapter 4 this is so overwhelming, that solutes behave as hydrophilic zwitterionic salts, even though they bear substantial apolar groups. The observation of intramolecular destructive overlap of hydration shells in α-amino acids is not new (see Chapter 1). However, to our knowledge, what has not been found previously, is that apolar group interactions can be additive inside and outside the influence of the hydrophilic hydration sphere. This additivity remains rather surprising. A gradually decreasing influence with increasing distance from the polar group would be anticipated because the effects of the polar (ionic) groups on the polarisation of surrounding water molecules are strongly distance-dependent. Apparently, it is the first layer of water molecules around polar groups which is distorted to such an extent that it influences the hydration of the first three attached CH_2-groups to a similar extent (*i.e*. all three CH_2-groups interact with the same influenced water molecules). The next water layer around polar groups is probably influenced as well, since the interactions involving the cosolutes which we investigated are electrostatic and therefore long-range, especially for the (zwitter)ionic cosolutes. This leads to the conclusion that either water in the second (and following) hydration shell(s) is not affected to such an extent that it prevents the formation of independently hydrated hydrophobic hydration shells or another method (than kinetics) needs to be employed to reveal such effects. The use of cosolutes with longer alkyl chains is also required in this respect. Unfortunately, these cosolutes will show increased tendency for the formation of hydrophobic aggregates.

Returning to the use of additivity schemes, it is important to mention that the independence of group interaction parameters on the presence of other functional groups in the same solute is too drastic an assumption of the additivity concept introduced by Savage and Wood more than 20 years ago[2]. The theory has been criticised in the literature as well[3]. On the other hand, the deviations from this simple additivity scheme elucidate the details in specificity of pairwise interactions in dilute aqueous solutions.

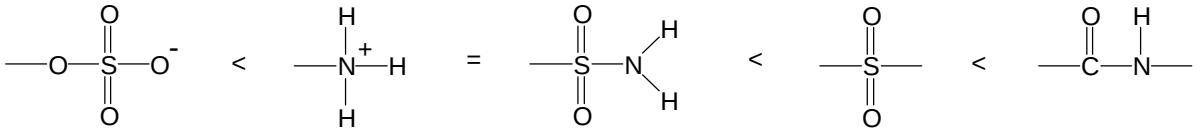
In summary, it is believed that additivity schemes should be used with particular care. Preferably, revisions should be made which take mutual intramolecular group interactions and stereochemistry into account.

Polar group contributions

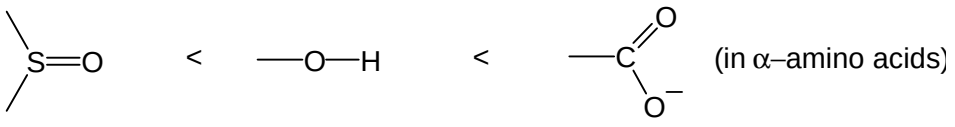
As has been demonstrated, solutes which are polyfunctional are heterogeneously hydrated (which, in fact, is almost every water-soluble molecule) and the different hydration shells within the solute interact and overlap, usually at the expense of the hydration of all of them. Not surprisingly, it appeared that the hydration of an ionic group is more incompatible with a hydrophobic hydration shell than is that of a polar nonionic group. This is due to stronger water-functional group interactions in the case of the ionic groups (*i.e.* ion-dipole versus dipole-dipole interactions) which gives rise to a hydration shell which is more difficult to disrupt.

It was interesting to find that, despite the hydrophilic character of the polar groups of all investigated cosolutes (including studies on solutes not described in this thesis[4]), the contributions of these groups to the kinetic medium effects on the hydrolysis of activated amides were usually negative (-CONH, $-SO_2NH_2$, $>SO_2$, $-OSO_3^-$, $-NH_3^+$) and positive only in the case of a hydroxyl (-OH), sulfoxide (>SO) and amino acid carboxylate ($-CO_2^-$) group. Since the activated complex (AC) is more polar than the initial state (IS), interactions of the polar groups with the AC are anticipated to be more favourable leading to positive contributions to G(c). However, this is too simplistic a view.

Although sometimes no exact values for the Gibbs energy of interaction of the polar groups could be determined, due to lack of additivity, it is possible to give reliable estimates. Subsequently, they can be ranked in order of contributions of the polar group X, G(X), to the kinetic medium effect. This provides information about the noncovalent interactions between the polar group and the IS and/or AC. Another possibility is to rank them in order of the apparent hydrophobicity of the attached CH_2-groups (reflected by the $G(CH_2)$ value), which gives information about the interactions between the individual hydration shells within the cosolute. Ranking the retarding polar groups in order of increasing G(X), *i.e*. in less retarding order, leads to the following sequence:



For the polar groups that exert an accelerating effect on the activated amide hydrolyses, the ranking order is (again in increasing G(X)):



In fact, the two series can be combined, yielding one series, the sulfate having the lowest G(X) (most retarding) and amino acid carboxylate group having the highest G(X) (the most accelerating) solute. The pattern of this series is not readily explainable. The ionic headgroups are the most retarding in the series with the exception of the α-amino acid carboxylate. When transition state stabilisation effects are taken into account the sulfate and ammonium groups behave unexpectedly. Explanations for their behaviour have been discussed previously[5,6].

Since the reactants (activated tertiary amides) are H-bond acceptors only and the activated complexes in transition states of their hydrolyses contain both H-bond acceptor and H-bond donor sites, due to the involvement of two water molecules, it is possible that the H-bonding abilities of the cosolutes are of importance in the interaction processes. The existence of a correlation between cosolute H-bond acceptor ability and G(X) would provide strong evidence for interactions between the cosolute polar groups and the activated complex. A correlation of the cosolute H-bonding donor ability can indicate interactions with both probe and activated complex.

One way to express H-bonding ability is by assessing the number of H-bond donor and acceptor sites in the solutes. This is indicative for the extent of hydration, *i.e*. the number of water molecules which can be restrained in their motions in the polar hydration shell due to directional association with the polar groups, but they do not necessarily reflect the strength of the interactions (*i.e.* Gibbs energies of polar group hydration). The numbers of H-bond donor and acceptor sites of the cosolute are plotted versus the G(X) values in Figure 6.1. As can be seen, a correlation with H-bond donor ability is not obvious. Apparently, H-bonding interactions between the reactant and the polar groups of the cosolutes do not play an important role. Large variations in G(X) are observed where H-donor capabilities are similar. A relationship with H-bond

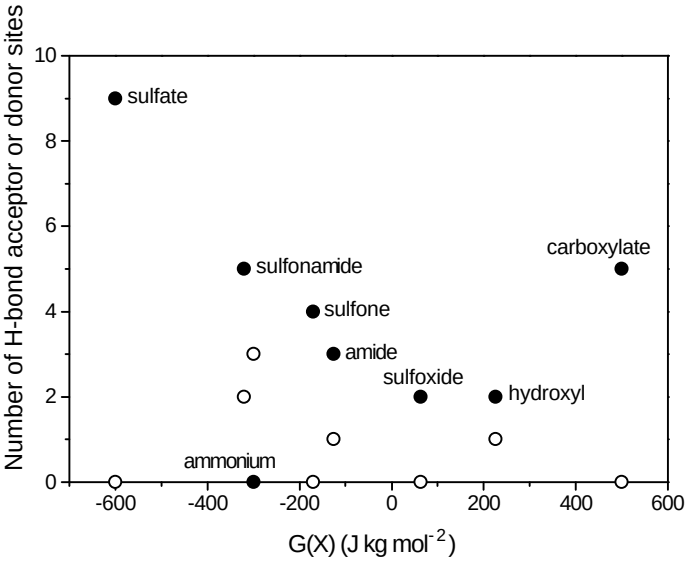


Figure 6.1 *Number of H-bond acceptor (●) and H-bond donor (O) sites of the polar group versus pairwise Gibbs energy polar group interaction parameters.*

acceptor ability, however, is
more likely to exist. The higher the number of H-bond acceptor sites of the polar group, the lower (more negative) the G(X). To put it in other words, the higher the ability of the polar group to accept H-bonds of surrounding water molecules the more the transition state is destabilised (increased in Gibbs energy), since most G(X) values are negative. There are two possible explanations. The first is the consideration of direct intermolecular H-bonding interactions, which require dehydration of the solutes. The retarding factor which causes the minus sign of the G(X) must then be attributed to the fact that dehydration costs more Gibbs energy than is gained by direct H-bonding interactions. Only for the sulfoxide and hydroxyl group presumably the gain in direct polar group-AC interactions dominates the unfavourable dehydration energy. The question remains why dehydration would take place in the first place. Maybe it is driven by other favourable noncovalent (hydrophobic?) interactions with the AC. In view of this explanation, direct H-bonding interactions would then also be operative in the more hydrophobic initial state. However, the IS is not a H-bond donor. Probably, the better explanation is that the possibilities of water to adapt the specific orientation which is required for the formation of the activated complex in the transition state are increasingly reduced when the hydration of the polar groups (in terms of number of H-bonding acceptor sites) increases[7]. The H-bonding interactions are highly directional and reduce the degrees of freedom of the water molecules involved in the interactions. Clearly, the number of H-bond acceptor sites of the polar group X is not the only factor which determines G(X), since deviations from the trend in Figure 6.1 are observed. The strength of the H-bonding interactions is also important. The kinetic medium effect of the ammonium group (a rather negative G(X) is observed, despite the absence of donor acceptor sites), has been explained in terms of electrostriction of water by which the possibilities for water to find the required orientation for the formation of the transition state are further attenuated. The positive contribution of the carboxylate group was explained in terms of water polarisation effects, *i.e.* very strong H-bonding interactions with water. How much do the G(X) values reflect apolar group hydration and vice versa? It is anticipated that a relationship between the G(X) and the $G(CH_2)$ parameters exists, since the intramolecular hydration shells overlap. That is, when the polar groups are more extensively hydrated, the destructive effect on the apparent hydrophobicity of adjacent apolar groups will possibly be larger. In order to investigate the occurrence of such a relation, the values of $G(CH_2)$ have been plotted versus the G(X) values in Figure 6.2. From this figure it appears that there is no clear relationship between the two pairwise group interaction parameters, although cosolutes with ionic polar groups reduce the apolar

contribution to medium effect more than do nonionic polar groups, due to the stronger hydration of the first. The sulfate, ammonium and amide polar groups have rather different G(X) values, but the $G(CH_2)$ values for the alkyl groups are rather similar. The apparent correlation between the molecules possessing a sulfonamide, a sulfone or an alcohol group cannot be explained by decreased hydration in terms of H-bond acceptor sites. The number of H-bond acceptor sites of these polar groups decreases in the order sulfonamide (5) > sulfone (4) > alcohol (2). If this is interpreted as decreased hydration, than the hydration shells of the CH_2-groups in the alcohols are the least perturbed by the polar group hydration and are therefore more available for hydrophobic interactions resulting in a more negative $G(CH_2)$. The opposite, however, is observed; the alcohols show *less* negative $G(CH_2)$ values. The same holds for the series with sulfonamide, sulfone and sulfoxide polar groups. Apparently, the number of hydrogen bonds which can be accepted by the polar functionality cosolute is not an adequate measure for its hydration. It should be noted that the donating RO–H···O (water) hydrogen bonds of the alcohol are stronger than the hydrogen bonds involved in the sulphur cosolutes, which might be of importance. Also, differences in the structure of the hydrogen bonds[8] might influence the observed trends. It is of interest to plot the results versus the Gibbs energies of polar group hydration, if available. Some important characteristics of the interactions between the polyfunctional molecules and the initial state and transition state of BT and BPhT undoubtedly have gone unnoticed by the present experimental data. The inclination exists to always view interactions as favourable (stabilising) for otherwise there would not be any interaction. However, unfavourable (destabilising) interactions (such as apolar-polar interactions) can be

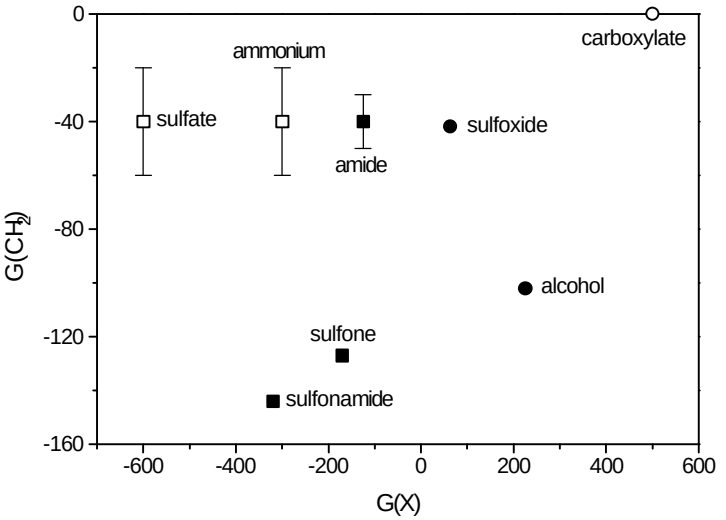


Figure 6.2 *$G(CH_2)$ versus G(X) (in J kg mol^{-2}) for polyfunctional solutes with short alkyl chains (up to 3 C-atoms) obtained from kinetic medium effects on the hydrolysis of BPhT (and BT in the case of the sulfate and ammonium groups). The G(X) value for the carboxylate group has a large error and possibly extends to higher values (see Chapter 4). Circles represent accelerating solutes, squares retarding solutes. Open symbols represent*

enforced and little or no attention has been paid to those possibilities.

ionic solutes. Error bars result from lack of group additivity.

It is clear that kinetic solvent effects cannot answer all these questions, partly because interactions with two states take place. However, they provide accurate data for solutes which differ only slightly in structure and therefore give a good indication about which interactions play a dominant role in the molecular recognition processes.

6.3 α-Amino acid hydration: Improved understanding of protein hydration?

In Chapter 4 it was indicated how far-reaching the effects of the zwitterionic α-amino acid ammonium and carboxylate hydration shells are on, particularly, the hydration and noncovalent interactions involving the side chains. Of course, these results are not immediately transferable to the hydration of a protein. The α-amino acid residues in proteins are linked by polar, but nonionic, peptide groups, *i.e*. the protein α-amino acids are not zwitterionic. Therefore, the impact of the polar hydration on the apparent hydrophobicity of the side chains is reduced considerably. In addition, the compact native structure of a protein causes many α-amino acid residues in the protein interior to interact solely with each other, without the interference of water. However, although many α-amino acid residues are 'buried' (*i.e*. not exposed to the solvent water) in the folded protein, there are a number of hydration sites, such as the active site of an enzyme or a hydrophilic cleft, which can be extensively hydrated[9]. Also, in the energetics of protein unfolding, the destructive overlap of polar (non)ionic group hydration and apolar hydration shells certainly plays a role, since 'buried' residues are brought into contact with water. Therefore it is relevant to obtain insight into the aspects of α-amino acid (side chain) hydration. We highlighted some of those aspects. Though the zwitterionic α-amino acids might not be the best model compounds, they are certainly not worse than α-amino acids which are protected on both termini. Moreover, from the kinetic solvent effects, Gibbs energies of interaction are obtained which are in many aspects more informative than the enthalpies of interactions which have been so often determined.

Studies of the hydration of small peptides are the next obvious step. Preliminary investigations involving peptides were encouraging in the sense that the kinetic solvent effects show a clear side-chain dependency and research along these lines will be continued.

6.4 Chiral recognition in aqueous solution

A topic which has not been dealt with experimentally in this thesis but which needs at least some attention is the chirality of the α-amino acids. In Chapter 1, it was seen that chiral recognition between zwitterionic α-amino acids in aqueous solution can be established.

Several attempts have been made to investigate the possibility of diastereomeric noncovalent interactions between α-amino acids and other chiral molecules in dilute aqueous solution by means of kinetic medium effects. A chiral activated amide has been synthesised and one of its optically pure enantiomers was hydrolysed in the presence of α-amino acids enantiomers and chiral alcohols. On the basis of the SWAG-approach, diastereomeric interactions are not expected to give different results; the molecules interact through the same functional groups. However, it is possible that the reactant and solute molecules require specific positioning to be able to interact and this may be achieved more effectively by one diastereomeric couple than by the other[10]. This accurate positioning also seems to be important in a recent study dealing with enantioselective Lewis-acid catalysis of Diels-Alder reactions in water in the presence of catalytic amounts of α-amino acids[11]. An issue that needs consideration is that if the solute-solute interactions take place via overlap of hydration spheres, the chirality must be sensed through several layers of water molecules. Recently, theoretical evidence for the existence of chiral water trimers was obtained[12].

These observations are encouraging for performing a kinetic study along these lines. Though differences in reactivity have been observed in a preliminary study, their relevance for establishing diastereomeric interactions in aqueous solution needs further attention.

6.5 Acknowledgements

Adriana Kertelj and Frans Wessels are thanked for their perseverance to measure diastereomeric interactions in aqueous solution by means of kinetic medium effects.

6.6 References

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Summary

Noncovalent interactions constitute the main driving force for the formation of many biological structures in aqueous solution. A clear example is the water-mediated formation of the folded state of proteins, which is largely driven by hydrophobic interactions between apolar α-amino acid side chains. But there are many other noncovalent interactions contributing to their stability, such as peptide-peptide H-bonding and salt-bridges. It is very difficult to obtain the magnitudes of the energetic contributions of these noncovalent interactions to the stability of the protein from studies of the entire protein molecule. Therefore, investigations have been focused on the building blocks of proteins, the α-amino acids, particularly on their hydration properties. There are two approaches by which information about hydration properties can be obtained:

1) By studying ideal solutions, *i.e*. where the solute is at infinite dilution.
2) By studying deviation from nonideal behaviour, *i.e*. where solute-solute interactions take place. These are mediated by the solvent, since the solutes interact via overlap of their hydration shells.

Both approaches have led to improved insights into the hydration of biological molecules. The second approach is advantageous because it bears relevance to molecular recognition processes in (bio)chemistry. Solute-solute interactions can be expressed in pairwise thermodynamic interaction parameters. For most solutes, however, mainly pairwise enthalpies of interaction have been obtained. Enthalpy is an informative thermodynamic quantity, but by itself says little about whether the interaction is favourable or not (in terms of Gibbs energies). In this thesis, the primary aim was to obtain information about the Gibbs energies of pairwise solute-solute interactions involving small biological or biologically relevant molecules containing both apolar and polar moieties, where the apolar moiety is varied in size while the polar moiety remains unchanged. These solutes included zwitterionic and anionic α-amino acids, small peptides, small cyclic amides and alkylated ammonium bromides. Interaction parameters were obtained by measuring kinetic medium effects of small amounts of these solutes on (water-catalysed) hydrolytic processes in dilute aqueous solution. The solutes interact with the initial and the (polarised) transition states of the reactions in a stabilising or destabilising way, thereby changing the Gibbs energies of activation for the hydrolytic process. The kinetic results were converted into pairwise Gibbs energy interaction parameters, G(c) values, using a previously developed theory.

The importance of obtaining information about the hydration of the zwitterionic α-amino acids and small peptides has been emphasised. Since they are polyfunctional molecules, which have clearly distinct hydration shells within the molecule, it was anticipated that overlap of these intramolecular hydration shells will affect the interactions with other solutes (here the IS and TS of the hydrolysis reactions). Since the contributions of the carboxylate and the ammonium group, as well as those of the side chains, were difficult to distinguish, also solute-solute interactions involving anionic α-amino acids (amino in stead of ammonium group), alkylated ammonium bromides (cationic ammonium group only) and cyclic peptides (amide group only) were assessed. In this way, an attempt was made to obtain a unified picture of hydrophilic and hydrophobic hydration, of the interactions between hydrophilic and hydrophobic hydration shells within one solute molecule and of the implications for solute-solute interactions in aqueous solution.

In Chapter 2, the effects of *N*-alkyl-2-pyrrolidinones with varying alkyl chains and several related compounds on the water-catalysed hydrolysis reactions of two activated amides (1-benzoyl-(3-phenyl)-1,2,4-triazole) and an activated ester (*p*-methoxyphenyl dichloroacetate) are reported. The solutes induced a retardation of the reactions, indicative of dominant stabilising hydrophobic interactions with the IS of the reaction. Two important features emerged from the G(c) values:

1) The kinetic effects were clearly a combination of IS and TS effects as revealed by a comparison of the results of different kinetic probes and by some ester analogues of the pyrrolidinones. Polar amide-amide and amide-ester interactions appeared to play an important role in addition to hydrophobic interactions.
2) Hydrophobic interactions of the alkyl group attached to the amide nitrogen atom are reduced considerably by the amide hydration up to the γ-carbon atom. Remarkably, additivity in the contribution of these CH_2-groups to the kinetic medium effect was observed (*i.e*. they contributed equally to the G(c)), though this contribution was much smaller than that for more remote apolar units. Additivity in group interactions was expected for more remote CH_2-units as well, but insufficient data were available to confirm this.

Chapter 3 deals with the retarding kinetic solvent effects on the water-catalysed hydrolysis of 1-benzoyl-1,2,4-triazole, caused by four series of differently alkylated ammonium bromides. A similar picture to that observed in Chapter 2 emerged, but in a more pronounced way. In the tetraalkylammonium bromides, the apparent hydrophobicities of the first two methylene moieties are even more reduced because the ammonium cation is more strongly (but not necessarily more extensively) hydrated than the polar tertiary amide group (ion-dipole vs. dipole-dipole interactions). The other alkylated ammonium bromide series showed clearly

the trend which was anticipated in Chapter 2, namely, that additivity of CH_2-group interactions takes place outside the influence of the hydration sphere of the polar/ionic moiety.

In Chapter 4, the outstanding hydration properties of zwitterionic α-amino acids are identified. The important conclusions are enumerated. First, these solutes generally accelerated the water-catalysed hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole. Strong evidence was obtained against the operation of general-base catalysis by the carboxylate group. However, the data clearly indicate that the carboxylate group is the functional group responsible for the accelerations. The accelerations are brought about by polarisation of the water molecules involved in the transition state as induced by the carboxylate group, by which the kinetic basicity of water is enhanced. Second, the (apolar) side chains generally did not interact significantly with the IS and the activated complex in the TS. The chains were camouflaged by the strong hydration of the ionic groups which extended to at least four consecutive C-atoms. Unfortunately, the limited water solubility of most α-amino acids prevented the investigation of interactions involving CH_2-moieties more remote from the polar group. It became clear that the hydration shell of the ionic groups is, albeit dominating, not *pre*dominating. The destructive overlap of incompatible hydrophilic and hydrophobic hydration shells is mutual and also at expense of the ionic hydration. Third, the remarkable observation was made that phenylalanine and 3-phenylserine are the only two α-amino acids among the investigated ones which *retard* the hydrolysis reaction. Obviously, the noncovalent solute-IS/TS interactions are not governed by the zwitterionic appearance of the solute in the case of these α-amino acids. Kinetic medium effects on differently substituted activated amides led to the conclusions that stacking interactions between aromatic groups are not involved and that hydrophobic interactions largely dominate these effects.

To study the kinetic medium effects of anionic α-amino acids (*i.e*. at high pH) and some small peptides, another kinetic probe was required, since the mechanism of the hydrolysis reactions of the activated amides used in Chapters 2-4 changes at higher pH values. The S_N1-type hydrolysis of 2-(4-nitrophenoxy)tetrahydropyran was studied instead (Chapter 5). Notwithstanding the fact that problems arise upon comparing the results with those from Chapter 4, the kinetic medium effects by itself revealed interesting features of α-amino acid and peptide hydration.

Chapter 5, where kinetic medium effects of anionic α-amino acids on the hydrolysis of the acetal are described, showed that the combination of the hydration shells of the amino (uncharged) and carboxylate groups was insufficient to completely overwhelm interactions involving the side chains. An excellent

correlation is observed between the number of CH-groups in the side chain and the ln(k) observed at 1 molal of solute, up to n(CH) = 6, which again corresponds with the γ-carbon atom. α-Amino acids with side chains containing more than 6 CH-groups also showed a linear correlation, but with a higher slope, which is in fact similar to that obtained for linear alcohols. This supported the conclusions drawn in Chapters 2 and 3, namely that pairwise CH_2-group interactions can be additive inside as well as outside the influencing hydrophilic hydration sphere of the polar (ionic) group and that the hydration shell of the investigated polar (ionic) group extends to the third carbon atom remote from this group. Moreover, clear differences between kinetic medium effects caused by isomers showed up, for example for Val/nVal and Leu/Ile and for the dipeptides Gly-Val and Val-Gly, which could be explained by apolar side chain branching within or outside the polar hydration shell and by the presence of a side chain away or in the vicinity of the carboxylate groups, respectively. Remarkably, diglycine showed a rate acceleration, whereas tri- and tetraglycine showed rate retardations. Explanations for these effects have been offered.

From the kinetic medium effects a unified picture emerged on how incompatible hydration shells within one solute molecule overlap destructively. Generally, hydrophilic hydration appears to overshadow hydrophobic hydration up to the third carbon atom remote from the hydrophilic group, which corresponds with 1-1½ layers of water molecules in the hydration shell. The dominance of hydrophilic over hydrophobic hydration is caused by two factors. First, the hydrophilic group-water interactions are stronger than the hydrophobic group-water interactions. Second, the water-water H-bonding interactions around hydrophilic groups is changed compared to the bulk. Hydrophilic ionic groups tend to increase the *local* density of water, *i.e.* cause solvent electrostriction. Hydrophilic polar groups also affect H-bonding between surrounding water molecules. Hydrophobic groups, on the contrary, do not affect the H-bonding properties of their hydration water significantly. Consequently, the effects of the hydrophobic hydration water on intermolecular interactions is relatively easy attenuated by hydrophilic hydration.

The results emphasise the importance of consideration of intramolecular hydration overlap effects in studies of intermolecular noncovalent interactions in water of almost every kind (including studies involving protein hydration and protein folding), since almost every molecule which dissolves in water is polyfunctional and therefore heterogeneously hydrated.

Samenvatting

Niet-covalente interacties vormen de basis voor de vorming van veel biologische structuren in waterige oplossing. De vorming van de compacte 3-dimensionale structuur van eiwitten is het primaire gevolg van hydrofobe interacties tussen de apolaire zijketens van de aminozuren. Er zijn echter nog andere niet-covalente interacties die bijdragen aan de stabiliteit van het gevouwen eiwit, zoals waterstofbruggen, bijv. tussen peptide groepen, en zoutbruggen tussen geladen aminozuurzijketens. Het is onduidelijk wat de bijdragen zijn in termen van Gibbsenergieën van de verschillende niet-covalente interacties aan de stabiliteit van het eiwit, met name omdat het niet eenvoudig is het gehele eiwit te bestuderen. Daarom zijn veel onderzoekingen gericht op de bouwstenen van de eiwitten: de α-aminozuren, waarbij de interesse vooral uitgaat naar hun hydratatie-eigenschappen. Er zijn twee lijnen voor dit onderzoek naar hydratatie-eigenschappen:

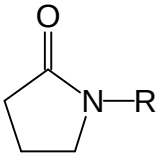
1) De studie van ideale oplossingen, waarbij het α-aminozuur oneindig verdund is en interacties tussen α-aminozuren en andere opgeloste deeltjes geen rol spelen.
2) De studie van afwijkingen van ideaal gedrag, waarbij interacties tussen α-aminozuren en andere opgeloste deeltjes plaats vinden en informatie verschaffen over de waterige omgeving van het α-aminozuur. Deze interacties worden sterk bepaald door overlap van hydratatieschillen.

De tweede benadering heeft als voordeel dat de resultaten relevant zijn voor moleculaire herkenningsprocessen in water in de (bio)chemie. Op deze manier kunnen paarsgewijze thermodynamische interactieparameters bepaald worden. Voor zwitterionische α-aminozuren zijn vrijwel alleen enthalpische interactieparameters gemeten. Hoewel de enthalpie een informatieve thermodynamische parameter is, wordt er geen inzicht verkregen in de vraag of de interactie gunstig is of niet (in termen van Gibbsenergie). Het onderzoek vastgelegd in dit proefschrift had tot doel informatie te krijgen over Gibbsenergieën van interactie waarbij kleine biologische of biologisch relevante moleculen betrokken zijn en wel zwitterionische en anionische α-aminozuren, kleine peptiden, kleine cyclische amiden en gealkyleerde ammoniumbromides. De interactieparameters werden verkregen via kinetische oplosmiddeleffecten van de te bestuderen moleculen op (watergekatalyseerde) hydrolysereacties in verdunde waterige oplossingen. De toegevoegde deeltjes hebben interacties met de hydrofobe begintoestanden en de gepolariseerde overgangstoestanden van de reacties, waardoor de Gibbsenergie van activering (en dus de snelheid) van het proces wordt beïnvloed t.o.v. de reactie in water. De kinetische resultaten werden omgezet in

paarsgewijze Gibbsenergie-interactieparameters (G(c)-waarden) m.b.v. een theorie die al eerder ontwikkeld was.

Het belang van de hydratatie van α-aminozuren en peptides is al benadrukt. Dit zijn polyfunctionele moleculen met duidelijk verschillende hydratatieschillen van de functionele goepen binnen het molecuul. Het onderscheiden van de afzonderlijke bijdragen van de functionele groep interacties aan de G(c)-waarde bleek complex. Daarom zijn ook interacties bestudeerd waarbij anionische aminozuren (met een neutrale aminogroep in plaats van een kationische ammoniumgroep), gealkyleerde ammoniumverbindingen (een kationische ammoniumgroep) en *N*-alkyl-2-pyrrolidinonen (amidegroep) betrokken zijn. Op deze manier werd een beter inzicht verkregen in hydrofiele en hydrofobe hydratatie, hoe de onverenigbare hydrofiele en hydrofobe hydratatieschillen binnen één molecuul overlappen en wat de gevolgen daarvan zijn voor intermoleculaire interacties in waterige oplossing.

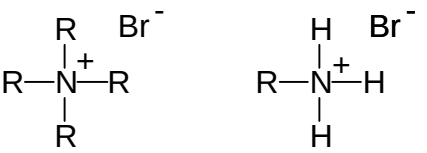
In Hoofdstuk 2 zijn de kinetische effecten van *N*-alkyl-2-pyrrolidinonen (zie afbeelding) met verschillende alkylketenlengtes en verschillende structureel gerelateerde verbindingen op de pH-onafhankelijke hydrolyse van twee geaktiveerde amiden (1-benzoyl-(3-fenyl)-1,2,4-triazool) en een geaktiveerde ester (*p*-methoxyfenyl-dichlooracetaat) beschreven. De *N*-alkyl-2-pyrrolidinonen vertragen de reacties,



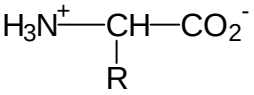
hetgeen indicatief is voor een dominante stabilisatie van de begintoestand d.m.v. hydrofobe interacties met het apolaire fragment van de toegevoegde deeltjes. Twee belangrijke conclusies volgden uit de analyse van de G(c)-waarden:

1) De kinetische effecten waren een combinatie van effecten op zowel begin- als overgangstoestand. Dit werd met name duidelijk door vergelijking van de resultaten van verschillende kinetische substraten en door resultaten van ester analoga van de pyrrolidinonen. Polaire amide-amide en ester-amide interacties bleken een niet onbelangrijke rol te spelen naast de hydrofobe interacties.
2) De hydrofobe interacties van de alkylgroep aan het amidestikstofatoom zijn aanzienlijk gereduceerd door de amidehydratatie tot aan het γ-koolstofatoom. M.a.w., de hydrofiele hydratatie domineert de hydrofobe hydratatie voor die methyleengroepen. Opmerkelijk was dat additiviteit in de bijdrage van deze methyleengroepen aan het kinetisch oplosmiddeleffect werd waargenomen, d.w.z. dat deze CH_2-eenheden eenzelfde bijdrage aan de G(c) leverden, hoewel deze bijdrage kleiner was dan voor verder weg gelegen CH_2-eenheden. Additiviteit in CH_2-groepinteracties werd ook voorspeld voor deze verder weg gelegen eenheden, maar er waren te weinig experimentele gegevens om dit te bevestigen.

Hoofdstuk 3 behandelt de vertragende kinetische oplosmiddeleffecten op de watergekatalyseerde hydrolyse van 1-benzoyl-1,2,4-triazool, veroorzaakt door vier series van verschillend gealkyleerde ammoniumbromides (voor voorbeelden, zie afbeelding). Overlap van hydratatieschillen bleek ook een rol te spelen bij de intermoleculaire interacties met deze toegevoegde deeltjes. In de serie van tetraalkylammoniumbromides was de ogenschijnlijke hydrofobiciteit van de eerste twee methyleengroepen gebonden aan het stikstofatoom van de ammoniumgroep nog geringer dan voor de eerste methyleengroepen van de *N*-alkyl-2-pyrrolidinonen. Dit benadrukt de sterkere (maar niet noodzakelijkerwijs verder uitgestrekte) hydratatie van het ammoniumkation in vergelijking met de tertiare amidegroep (ion-dipool versus dipool-dipool interacties). Resultaten m.b.t. de andere gealkyleerde ammoniumbromide series gaven aan dat additiviteit van CH_2-eenheden ook buiten de invloedssfeer van de hydratatie van de polaire (ionische) groep optreedt.



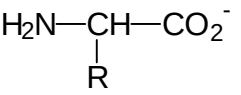
In Hoofdstuk 4 worden de hydratatie-eigenschappen van zwitterionische α-aminozuren (zie afbeelding) belicht. Deze toegevoegde deeltjes bleken over het algemeen de watergekatalyseerde hydrolyse van 1-benzoyl-3-fenyl-1,2,4-triazool te versnellen. De versnelling moet worden toegeschreven aan niet-covalente interacties met de carboxylaatgroep. Algemene basekatalyse bleek niet op te treden. Het wordt verondersteld dat de versnellingen teweeg worden gebracht door polarisatie van de watermoleculen in de overgangstoestand, geïnduceerd door de carboxylaatgroep, waardoor de kinetische basiciteit van het water toeneemt. De apolaire zijketens vertonen niet of nauwelijks interactie met de begin- (en overgangs)toestand van de reactie. De sterke hydratatie van de ionische groepen maakt de hydrofobe hydratatie van de zijketens vrijwel onmogelijk tot in ieder geval de eerste 4 CH_2-eenheden. De beperkte oplosbaarheid van de α-aminozuren liet de studie van de interacties van verder gelegen CH_2-eenheden niet toe. Hoewel de ionische hydratatie de intermoleculaire interacties domineert, wordt deze wel degelijk door de zijketenhydratatie beïnvloed. De destruktieve overlapping van hydrofiele en hydrofobe hydratatieschillen is wederzijds en gaat ook ten koste van de ionische hydratatie. Opmerkelijk is dat fenylalanine en 3-fenylserine de hydrolyse vertragen. In deze gevallen is het dus duidelijk niet de zwitterionische hydratatie die het kinetische oplosmidddeleffect domineert. Uit kinetische oplosmiddeleffecten gemeten voor andere substraten wordt geconcludeerd dat aromatische ‘stacking’-



interacties geen rol spelen en dat het vooral hydrofobe interacties zijn die het effect domineren.

Om kinetische oplosmiddeleffecten van anionische α-aminozuren (bij hogere pH) te bestuderen is een andere hydrolysereactie gebruikt omdat het mechanisme van de reacties beschreven in de Hoofdstukken 2 t/m 4 bij hoge pH verandert. Gekozen is voor de S_N1 hydrolyse van 2-(4-nitrofenoxy)tetrahydropyran (Hoofdstuk 5). Hoewel een vergelijking van de resultaten met Hoofdstuk 4 problemen oplevert, met name omdat water in Hoofdstuk 5 een veel minder belangrijke rol speelt in het aktiveringsproces, onthulden de oplosmiddeleffecten interessante hydratatie-eigenschappen van α-aminozuren en peptiden.

De kinetische oplosmiddeleffecten van anionische α-aminozuren (zie afbeelding) en enkele korte peptides op de hydrolyse van het acetaal, welke zijn beschreven in Hoofdstuk 5, werden niet volledig gedomineerd door de polaire (ionische) groepen en interacties met de aminozuurzijketens waren meer uitgesproken. Er werd een uitstekende correlatie gevonden tussen het aantal CH-eenheden (n(CH)) in de alkylzijketen en de ln(k) die waargenomen werd bij een α-aminozuurconcentratie van 1 molal tot aan (CH) = 6, hetgeen opnieuw overeenkomt met het γ-koolstofatoom. α-Aminozuren met meer dan 6 CH-eenheden in de zijketens lieten ook een lineaire correlatie zien, echter met een duidelijk grotere helling (grotere CH-bijdrage aan het oplosmiddeleffect), die gelijk was aan de helling voor lineaire alcoholen. Deze waarnemingen ondersteunen de conclusies die in de Hoofdstukken 2 en 3 zijn getrokken, nl. dat paarsgewijze interacties van CH-groepen zowel binnen als buiten de hydratatieschil van de polaire (ionische) groep additief kunnen zijn en dat de hydratatieschil van de onderzochte polaire (ionische) groepen zich uitstrekt tot het van deze groep afgelegen derde C-atoom. Verder werden duidelijke verschillen waargenomen tussen kinetische oplosmiddeleffecten veroorzaakt door isomeren, zoals voor Val/nVal en Leu/Ile en voor de dipeptides Gly-Val en Val-Gly. Deze werden verklaard door, respectievelijk, alkylketenvertakking binnen of buiten de hydrofiele hydratatieschil en de aanwezigheid van een zijketen in de nabijheid van of verder weg gelegen van de geladen carboxylaatgroep. Het was opmerkelijk dat diglycine de acetaalhydrolyse versnelde terwijl tri- en tetraglycine vertragingen veroorzaakten. Verklaringen voor deze waarnemingen werden aangevoerd en de resultaten geven aanleiding tot meer onderzoek naar peptidehydratatie.



De kinetische oplosmiddeleffecten leveren een beeld op van de manier waarop onverenigbare hydratatieschillen binnen de bestudeerde moleculen op een destructieve manier overlappen. Over het algemeen domineert hydrofiele hydratatie over hydrofobe hydratatie en wel tot het γ-koolstofatoom. Dit komt overeen met 1 tot

1½ laag van watermoleculen in de hydrofiele hydratatieschil. De dominantie van hydrofiele over hydrofobe hydratatie is o.a. het gevolg van het feit dat de interacties tussen de hydrofiele groepen en water sterker zijn dan die tussen de hydrofobe groepen en water en dat tevens de interacties tussen watermoleculen in de hydratatieschillen van hydrofiele groepen veranderd is t.o.v. puur water. De dichtheid van water rond ionische groepen is vergroot door elektrostrictie. Daarnaast beïnvloeden hydrofiele polaire groepen de 3-D H-brugstructuur in de hydratatieschil. Echter, hydrofobe groepen hebben geen, of een gering, effect op het H-brugnetwerk van water in hun hydratatieschil.

De resultaten benadrukken het belang van effecten van overlappende intramoleculaire hydratatieschillen in studies van intermoleculaire niet-covalente interacties in water van eigenlijk elk type molecuul (inclusief de studies naar eiwithydratatie en eiwitvouwing), omdat ieder molecuul dat redelijk goed opgelost kan worden in water polyfunctioneel is en dus heterogeen is gehydrateerd.